PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12Q 1/68, C12P 19/34

A1

(11) International Publication Number: WO 00/56933

(43) International Publication Date: 28 September 2000 (28.09.00)

(21) International Application Number: PCT/US00/07783

(22) International Filing Date: 23 March 2000 (23.03.00)

(30) Priority Data:
60/125,839 24 March 1999 (24.03.99) US

(71) Applicant *(for all designated States except US)*: PRINCETON SEPARATIONS [US/US]; 920 Route 33, Fairfield Corporate Park, Bldg. 7, Freehold, NJ 07728 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: LEVISON, Derek [US/US]; 7 Burton Road, Jackson, NJ 08527 (US).

(74) Agents: SCOLA, Daniel, A., Jr.; Hoffmann & Baron, LLP, 6900 Jericho Turnpike, Syosset, NY 11791 (US) et al.

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CHEMICALLY REACTIVE PLANE–RIGIDIZED CYANINE DYES AND THEIR DERIVATIVES

(57) Abstract

The invention describes novel plane–rigidized cyanine near–infrared (NIR) fluorescent dyes, their methods of preparation and use in the non–isotopic labeling of biological molecules. Such dyes contain a central cyclic hydroanthraquinone framework which provides for a stable and rigid structure to ensure low reactivity with neighboring molecules, a sharp absorption band, a high quantum yield, and emissions in a region largely devoid of natural background auto–fluorescence. Such dyes further comprise variable end groups flanking the central cyclic framework, allowing formation of a number of different plane–rigidized dyes, each having a slightly different emission wavelength. Functional groups are provided for attachment to target biological molecules. Moreover, hydrophilic functional groups are provided where necessary to significantly increase the water solubility of the dye molecules.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

CHEMICALLY REACTIVE PLANE-RIGIDIZED CYANINE DYES AND THEIR DERIVATIVES

FIELD OF THE INVENTION

The present invention relates to plane-rigidized cyanine near-infra red (NIR) fluorescent dyes, their methods of synthesis and use in the labeling of biological molecules.

BACKGROUND OF RELATED TECHNOLOGY

Cyanine dyes are of increasing interest as near-infra-red absorbing and fluorescing chromophores. Cyanine dyes have distinct advantages over most other fluorescent markers widely in use. One common problem encountered in biochemistry is the fact that most proteins, lipids and nucleic acids autofluoresce in the same range as their fluorescent labels, thereby significantly reducing the sensitivity of diagnostic assay applications utilizing these dyes. To overcome this problem, the use of a chromophore emitting in a range with essentially no natural background autofluorescence would greatly increase detection sensitivity of the labeled molecule. Two of the most popular fluorescent dyes presently utilized as biomolecular labels are fluorescein and rhodamine. Their respective fluorescent emissions are approximately 530 and 618 nm. This fluorescent emission overlaps the natural fluorescent emission of most biological molecules. Their use is therefore limited by this factor.

The fluorescent emission of near-infra red (NIR) cyanine fluorophores, on the other hand, typically occurs in a region with very low background fluorescence, making them useful for labeling of biological moleucles. NIR cyanine fluorophores have the additional benefit of absorbing in a region between 700-850 nm, which corresponds closely with the 785 nm wavelengths of the relatively cost-effective and commercially available GaAlAs diode lasers. A number of laser applications of these dyes in biotechnology have already been proposed. Inclusive of these applications is automated DNA sequencing.

Existing NIR cyanine dyes in use today are not without their own disadvantages. They do have both high extinction coefficients and high quantum yields, both desirable characteristics, however, possess only moderate chemical and photo stabilities. Most existing

NIR cyanine fluorophores can be described as "non-rigidized." Due to the long inflexible central conjugated cyanine structure, these dyes have a tendency to twist and bend. Upon absorption of light, these dyes tend to form radicals, which in turn can react with adjacent molecules resulting in a total loss of fluorophore. Such phenomenon is referred to as photo bleaching. A further disadvantage is that these NIR fluorophores are for the most part large, bulky and very hydrophobic thus making them generally insoluble in aqueous solutions.

One key strategy in elimination of internal twisting and bending of the cyanine chromophore is the introduction of carbon bridges into the conjugated system. For example, this can be accomplished in the form of additions of 6-membered rings. The bridges eliminate most twisting and bending by forcing a rigid, planar arrangement, defined here as "plane-rigidized," upon the conjugated π bond system. In comparison with non-rigidized systems, plane-rigidized NIR fluorophores have higher photo stability and, due to increased steric hindrance around the fluorophore, higher chemical stability. Due to maximization of conjugation in the π-system imposed by the planarity, plane-rigidized NIR fluorophores also have sharper absorption bands and stronger fluorescence yields. Overall imposition of carbon bridges result in a significant increase in the quality and lifetime of the fluorophore of the molecule. A typical example of a mono-cyclically plane-rigidized NIR dye has been described in German Patent No. DE 4326466A1.

This dye has incorporated a single 6-membered ring to stabilize the conjugated polyene system, as well as an isocyocyanate functional group for the attachment of this label onto primary amino groups of proteins or functionalized nucleotides, for example. In addition, this design has incorporated two sulfate groups to increase the water solubility of this otherwise rather insoluble molecule.

Heilig and Luttke have previously published work on the synthesis of an even more rigidized cyanine dye, containing a central tri-cyclic hydroanthraquinone framework. These dyes, however, are very insoluble in water and contain no functionalities for use as a biomolecular label.

Of added benefit would be the creation of a number of functionalized, soluble, plane-rigidized dyes NIR cyanine dyes, each having a slightly different emission wavelength to allow for simultaneous tracking of more than one target molecule by providing each target

molecule with its own unique dye label. Thus the need for a new label for biomolecules can clearly be fulfilled by the creation of a class of rigidized NIR cyanine dyes with these properties and the low background fluorescence described in the foregoing discussion.

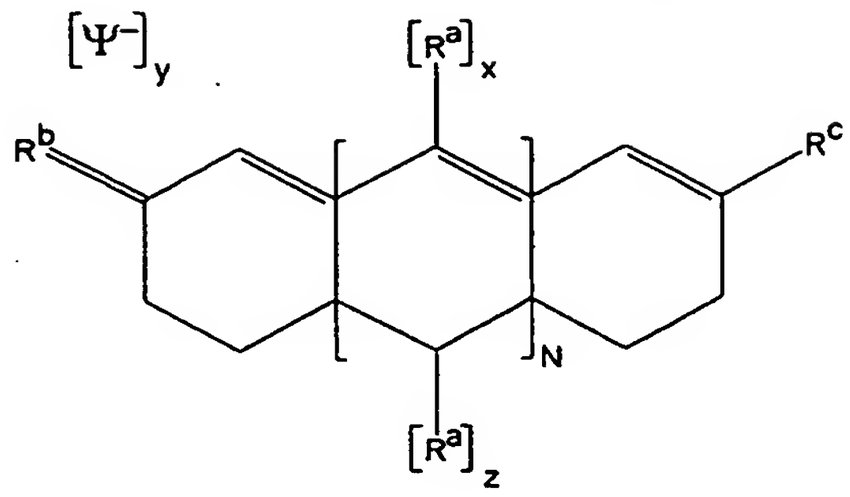
SUMMARY OF THE INVENTION

The present invention seeks to improve upon existing NIR plane-rigidized cyanine dyes by providing for dyes with a central cyclic hydroanthraquinone framework inclusive of a tri-cyclic framework as well as frameworks containing 4, 5 and 6 stabilizing 6-membered rings in the conjugated polyene system. Emission wavelengths will depend, at least in part, upon the number of stabilizing 6-membered rings within the hydroanthraquinone framework. Moreover, various end groups are described for each of the 3, 4, 5 and 6 ring -containing dyes. Such end groups flank the central planar network, allowing the formation of a large number of novel plane-rigidized cyanine dyes, each having a different emission wavelength. It will then be possible to label different target molecules each with its unique plane-rigidized NIR cyanine dye and to perform a single assay simultaneously for the target molecules with these probes mixed in the same solution, as for example in automated fluorescent- based DNA sequencing with dye-labeled dideoxynucleotide chain terminators. Moreover, functional groups are incorporated for attachment to target biological molecules and for increasing water solubility of the dye molecules when necessary.

It is noted that elongation of the central planar structure, that is of the hydroanthraquinone framework by addition of six-membered rings allows for an increase in the Stokes shift, offering a sharper absorption band with greater separation between the wavelengths of absorption and emission and longer wavelengths of absorption and emission, with less overlap with the natural fluorescent emission of most biological molecules. The result is a greater signal to noise ratio. The dyes of the present invention provide highly stable and rigid structures to ensure low reactivity with neighboring molecules, a sharp absorption band and high quantum yields.

DETAILED DESCRIPTION

In one aspect of the invention there is provided a central plane-rigidized cyanine dye for labeling of biological molecules wherein in the dye has the following formula:



wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

Because the number of rings comprising the central planar network can vary from 3-6 the emission wavelength of such dyes will also vary accordingly. Groups R^b and R^c which may be the same or different are conjugated to the central planar network. These groups allow the dye to fluoresce and also serve to alter the wavelength emission of the dye. Therefore, numerous dyes are possible depending both on which groups are placed at positions R^b and R^c as well as the number of rings present within the central hydroanthraquinone network. A reactive substituent for bonding of the dye to a biological molecule may be placed at any one of positions R^a, R^b or R^c. In some instances groups R^a, R^b and R^c may contain a linker portion between their attachment to the dye and the reactive substituent in order to reduce steric hindrance between the dye and a biological molecule.

When a linker is present, it may have a variety of structures inclusive of a linear or branched, cyclic or heterocyclic, saturated or unsaturated groups, wherein the length of the linker varies from between 1-20 carbons atoms. The linker can also include one or more atoms inclusive of nitrogen, phosphorus, oxygen and sulfur atoms. Moreover, the linker may contain any combination of ether, thioether, amine, ester or amide bonds. The linker may also contain one or more double or triple bonds and these bonds may include carbon-carbon, phosphorous-oxygen, phosphorous-sulfur, nitrogen-nitrogen, nitrogen-oxygen, as well as aromatic or heteroaromatic bonds.

In one embodiment the reactive substituent present on at least one of R^a, R^b or R^c for direct bonding of the dye to a biological molecule or indirect bonding of the dye to a

biological molecule via a linker can be a number of compounds, classes of compounds or reactive groups. The reactive substituent may also be selected from a number of classes of molecules inclusive of alcohols, aldehydes, thiols, reactive esters, acids, acid halides, sulfonyl halides, hydrazines, ketones, haloacetamides, amides, and azides. Moreover, the reactive substituent may be a hydroxyl, amido, carboxylic, or amino group. In reference to particular compounds, any one or more of the following may be used: phosphoramidite, isothiocyanate, isocyanate, monochlorotriazine, dichlorotriazine, mono- or di-halogen substituted pyridine, mono- or di-halogen substituted diazine, aziridine, hydroxsuccinimide ester, hydroxysulfosuccinimide ester and imido ester.

It is noted that the reactive substituent is most likely to form a bond with moieties largely present on biological molecules inclusive of, but not limited to, amino, hydroxy, thiol, carboxyl, aldehyde and ketone groups. For example, the terminal amino groups of proteins as well as amino groups on amino acid residues such as lysine, would be available for reactivity with a reactive substituent on the dye such as a carboxylic group for formation of an amide bond. Moreover, hydroxyl groups present on sugar residues can react with an OR group on the dye, where R is H, for example. As another example, a phosphoramidite functionality on the dye can react with a 3' OH group on DNA or an oligonucleotide to form a covalent bond. Even where one of these moieties does not naturally exist on the biological molecule, it may be possible to modify the biological molecule so as to contain one of the moieties. For example, a modified thymine phosphoramidite (amino modifier dT) or alternatively a 5' amino linker (5' amino modifier C_6) may be incorporated during synthesis of a DNA oligomer using standard beta-cyanoethyl phosphoramidite chemistry. An isothiocyanate moiety on the dye would be able to react with the amino group on the modified thymine base or terminal amino linker arm of the primer oligonucleotide to form a thiourea bond. As another example, an N-hydroxysuccinimide-ester funtionality on the dye can react with amino groups on natural or amino-modified biological molecules.

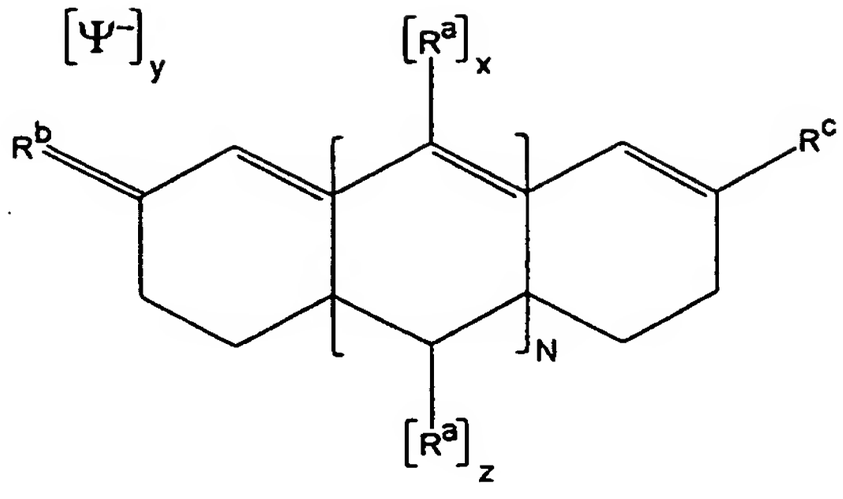
Although R^a may be a group containing a reactive substituent for bonding of the dye to a biological molecule, it may also be a non-reactive grouping present on the dye such as H or a substituted or unsubstituted aliphatic or aromatic group. The substituted or unsubstituted group may be from the classes inclusive of but not limited to alkyl, alkenyl, alkynyl, aryl and combinations thereof.

Depending on which groups are placed at positions R^b and R^c the dyes formed may be of an insoluble nature. Therefore, a polar moiety may be placed at one or more positions of R^a, R^b and R^c in order to increase the solubility of the dye in aqueous solutions. Polar moieties which may be placed at these positions are inclusive of, but not limited to, hydroxy, nitro, sulfonate, sulfate, carboxylate, substituted amine, quaternary amine and nitrile groups.

In one embodiment of the invention, R^b and R^c, which again may be the same or different, can specifically be O, OR, NR^1R^2 and CR^3, R^4, wherein R is H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, or a mono- or polysaccharide such as a sugar residue. R^1, R^2 and R^4 are independently H, substituted or unsubstituted alkyl, alkenyl, alkynyl, aryl or contain a reactive substituent selected from reactive substituents including, but not limited to: a number of classes of molecules inclusive of alcohols, aldehydes, thiols, reactive esters, acids, acid halides, sulfonyl halides, hydrazines, ketones, haloacetamides, amides, and azides. Moreover, the reactive substituent may be a hydroxyl, amido, carboxylic, or amino group. In reference to particular compounds, any one of the following may be used as the reactive substituent: phosphoramidite, isothiocyanate, isocyanate, monochlorotriazine, dichlorotriazine, mono- or di-halogen substituted pyridine, mono- or di-halogen substituted diazine, aziridine, hydroxsuccinimide ester, hydroxysulfosuccinimide ester and imido ester. R^3 is a ringed structure selected from the following: indole, oxazole, thiazole, imidazole, quinoline, isoquinoline, aniline, carbazole, benzindole, benzoxyazole, benaothiazole, benzimidazole, benzotriazole or substituted versions thereof. R^1 and R^2 may join together to form a ringed structure selected from the same group.

When such ringed structures are present, they may contain a reactive substituent selected from the same groups, classes of compounds or specific compounds aforementioned. Moreover, the ringed structures may carry a polar moiety as a substituent to increase the solubility of the dye in aqueous solutions. These polar moieties would be inclusive of, but not limited to, hydroxy, nitro, sulfonate, sulfate, carboxylate, substituted amine, quaternary amine, nitrile, sulfate groups and alcohols, In another aspect of the present invention, R^1, R^2 and R^4 may independently contain a polar moiety inclusive of, but not limited to, the same groups.

The present invention also describes the fluorescent complex which would be formed between a biological molecule which can be bound directly or indirectly to one or more di-molecules of the formula:

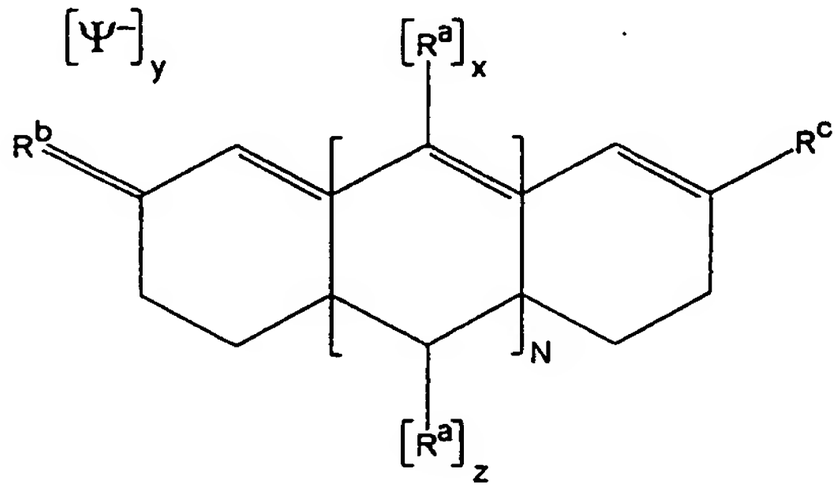


wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

The reactive functional groups present on either the dye or the biological molecule for formation of the complex are inclusive of, but not limited to, amines, amino group, hydroxy group, alcohols, thiols, acids, aldehydes and ketones. The biological molecule present in the fluorescent complex may be bound directly to the dyes via any combination of ether, thioether, amine, ester, amide, or a thiourea bonds. Wherein the biological molecule is bound indirectly to the dye via a linking group, the biological molecule would be bound to the linker via these same types of bonds. The attachment of the dye to a biological molecule, or for that matter to a linker, can include a nitrogen-containing group, a sulfur-containing group or an oxygen-containing group. One preferred bond which may be formed between the dye and the biological molecule is from the reaction of an activated ester of N-hydroxysuccinimide and a carboxylic acid. In this case the carboxylic acid is envisioned to be present on the biological molecule. For example, the terminal carboxyl group of a protein may be conjugated to this activated ester functionality. The biological molecule suitable for conjugation with the dye of the present invention may include proteins, nucleic acid polymers, polysaccharides, cells, as well as lipids. Wherein the biological molecule is a protein, the protein may be an antibody. Its presence within the fluorescent complex can allow for tracking of the antibody's reactivity with specific antigenic determinates using immuno assays as well as flow cytometric analysis for example. Wherein the biological molecule is a nucleic acid polymer, such polymers are inclusive of natural or synthetic oligonucleotides or oligonucleosides, chromosomes, DNA, as well as RNA.

A fluorescent complex may also be formed between the dye and a nucleotide or nucleoside base. One use of these fluorescent complexes would be in the incorporation of labeled bases within nucleic acids for purposes of sequencing.

A method of detecting a target biological molecule is also encompassed by the present invention. Such a method involves combining a sample that contains or is thought to contain a target molecule with a dye of the following formula:



wherein N=1-4; Ψ is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

Following incubation of the combined sample with the dye compound for a time sufficient for the dye to combine with the target molecule and form one or more dye-target molecule complexes that give a detectable fluorescent signal, the further step of detecting the signal and hence the target molecules, can be performed. The reaction conditions under which the target molecule may be directly conjugated with the dye compound may depend specifically on the nature of the biological molecule as well as on the solubility of the dye. However, standard procedures for labeling biological molecules exist. Among these is the procedure of Motsenbocker, et al. In general, when the reactive portion on the biological molecule is an amino group, the pH of the labeling buffer should be sufficiently basic to allow the amino group to maintain its reactivity for conjugation with the dye. The presence of solubility groups on the dye molecule allow it to remain in solution in such aqueous buffers.

Following incubation of the combined sample of the dye compound for formation of dye-target molecule complexes, it will be necessary to separate the fluorescent complex from

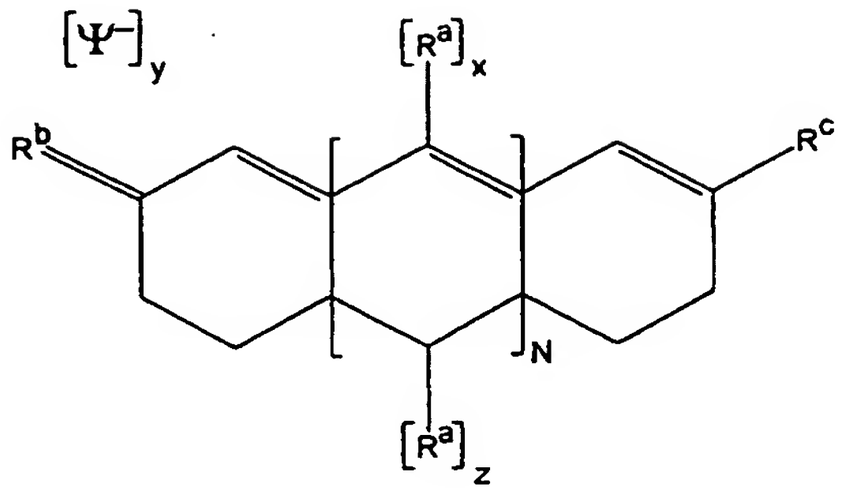
unbound dye. Such methods of separation are inclusive of gel filtration chromatography, HPLC reverse phase chromatography, as well as electrophoresis.

The target molecules useful for this method are inclusive of nucleic acid polymers, nucleic acid bases, proteins, lipids, cells, tissue specimens, and polysaccharides. The target molecule may be a nucleotide base inclusive of adenine, thimine, cytosene, guanine and other nitrogen heterocyclic bases. The target molecule may also be a polysaccharide (sugar), a natural or synthetic oligonucleotide or oligonucleoside, DNA, RNA as well as a chromosome. Wherein the target molecule is nucleic acid polymer, such as DNA it may be labeled by PCR incorporation of a dye labeled nucleotide base such as adenine, thymine, cytosine, guanine or other nitrogen heterocyclic bases within the DNA molecule. Alternatively, DNA or RNA can be labeled at the 3' position by reaction of the 3' OH group with a reactive substituent on the dye such as phosphoramidite. Wherein the R^c is defined as OR, conjugation with a target molecule which is a mono- or polysaccharide containing a reactive OH portion can be readily accomplished.

In another embodiment of the invention, the method just described may include the step of adding one or more reagents to the sample prior to or during the step of combining, where each additional reagent is capable of a response that is detectably different from the dye-target molecule complex. This further detection reagent may be an antibody, oligonucleoside or oligonucleotide, for example.

In yet another embodiment of the present invention, the aforementioned method may further comprise the step of adding one or more reagents to the sample during the step of incubating where each additional reagent is capable of a response that is detectably different from the dye-target molecule complex. Here again the further detection reagent may be inclusive of, but not limited to, antibodies, oligonucleosides, and oligonucleotides.

The present invention also covers a method of detecting a target molecule wherein a probe is provided which is capable of recognizing or reacting in a specific way with the target molecule of interest. In this method the probe is a fluorescent complex formed from a reaction of the probe with the dye molecule of the formula:

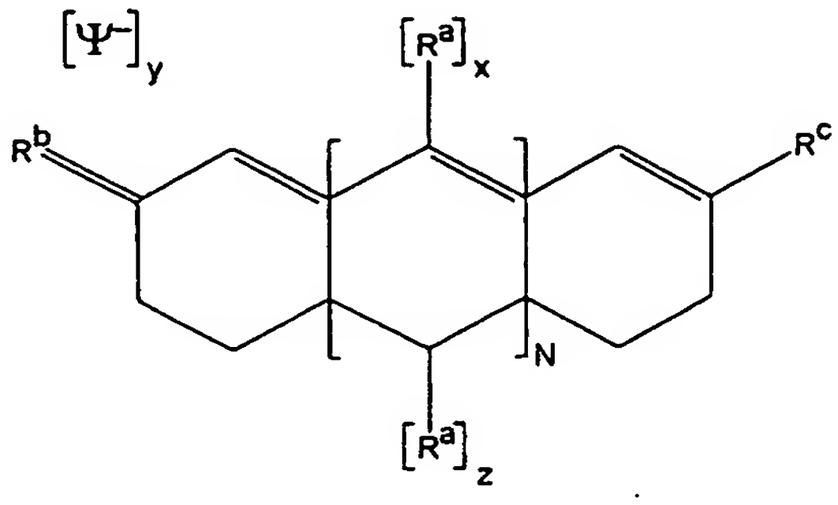


wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

A sample that contains or is thought to contain a target molecule is combined with the probe. The combined sample is thereafter incubated for a time sufficient to allow the target molecule to bind with the probe to form one or more dye-probe-target molecule complexes that give a detectable signal followed by detection of the signal. In this way the target molecule may be qualitatively or quantitatively detected. For quantitative analysis, standards would be required for comparison. The target molecule may again be selected from nucleic acid polymers, nucleic acid bases, proteins, lipids, cells, tissue specimens and polysaccharides. The target molecule may specifically be DNA or RNA, for example. The target molecule may also be a protein located within a cell or an antigenic determinant present on a cell. The probe may in this case be an antibody specific for such a protein or antigenic determinant which when bound thereto enables one to detect the molecules of interest. Wherein the target molecule is DNA, the probe may be an oligonucleotide complimentary in sequence to the DNA, as an example. As was aforementioned, it may be necessary to modify the oligonucleotide probe by incorporation of an amino modified base or a 5' amino linker in order to enable the oligonucloetide to be conjugated to the dye or, alternatively, the 3' OH group on the oligonucleotide can be conjugated to the dye, depdening on the dye's reactive substituent.

The present invention provides for a method of characterizing a nucleic acid sample. In this method one or more fluorescent complexes are provided wherein the complexes are

formed from a reaction of one or more biological molecules with at least one dye compound of the formula:



wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

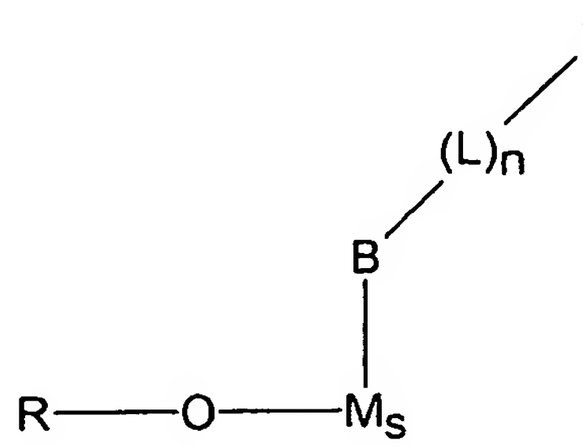
Following the combination of the nucleic acid sample with the fluorescent complexes the sample is incubated with the complexes under reaction conditions which would allow for the fluorescent complexes to combine with or incorporate within the nucleic acid to form a staining profile having detectable fluorescent signals. This staining profile would be characteristic of the sample. Biological molecules suitable for combination with the dye compound would include natural, synthetic or modified nucleotides, nucleosides, oligonucleotides, oligonucleosides, DNA, RNA or proteins. Most notably, this method may be applied to the sequencing of DNA wherein the fluorescent complexes incorporated within the DNA can be dideoxynucleotide triphosphate chain terminators bound to the dyes. Provided each dideoxynucleotide triphosphate is bound to a unique dye of the preceding formula with its own characteristic emission wavelength, the nucleic acid may be characterized with regard to its sequence when a standard Sanger method of DNA sequencing is followed. Using PCR amplification techniques, a heat-stable DNA polymerase is used to make a copy of the DNA template. By choice of an appropriate primer the region of the nucleic acid that is copied can be predetermined. DNA synthesis occurs by incorporation of unlabeled deoxynucleotide triphosphates and random termination of the chain occurs when the dideoxynucleotide fluorescent complexes are incorporated.

In this way a display of a continuous set of DNA fragments that differs in length by only one nucleotide can be obtained and the identity of the chemical nature of the end dideoxynucleotide triphosphate chain terminator may be readily identified during separation of the reaction products by electrophoresis on polyacrylamide gels with simultaneous monitoring of emission wavelengths corresponding to each of the dyes bound to the chain terminators. A laser diode is used for exciting the fluorophore and a photodiode is used for detecting the infrared emission. In one embodiment, therefore, the nucleic acid sample is a solution made up of nucleic acid polymers, in this case DNA fragments, separable by means of relative mobility. Characterization may be with respect to purity of the solution, composition of polymers in solution and composition of components present in solution.

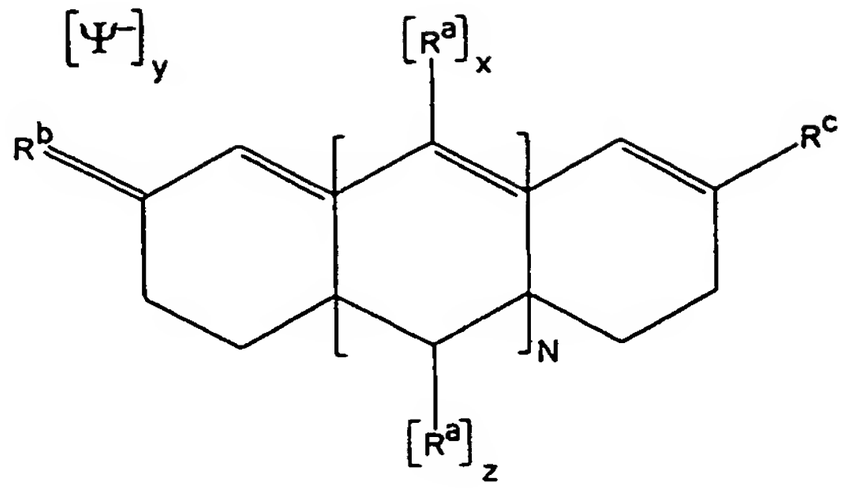
In another embodiment, the method of characterizing a nucleic acid sample may also involve adding one or more additional reagents to the sample wherein each additional reagent is capable of response that is detectably different from the fluorescent signal of the dye-nucleic acid complex. The additional detection reagent may be an antibody, for example.

Finally it is noted that the nucleic acid sample to be characterized can include not only DNA but RNA as well. When RNA is to be sequenced using the Sanger method, reverse transcriptase is used to make a DNA copy of an RNA template and the resulting DNA is sequenced by the same methods herein described.

Referring to the foregoing DNA sequencing method described, one embodiment of the present invention encompasses a chemical complex of the formula:



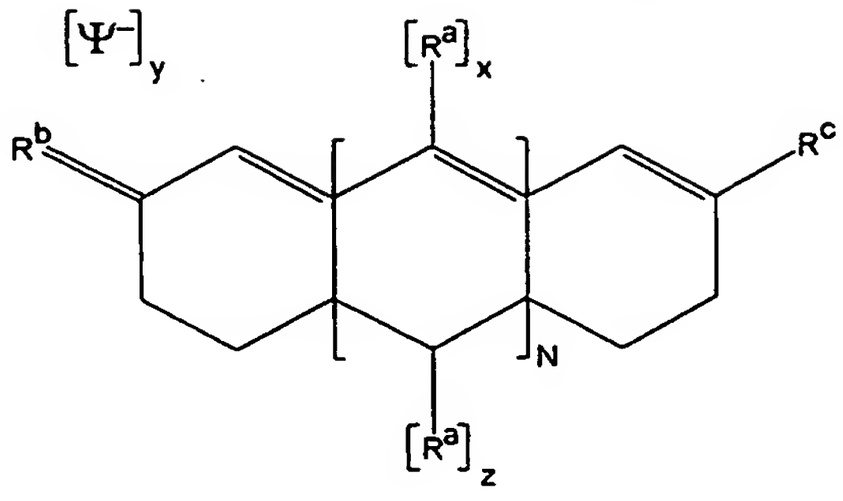
wherein D is of the formula:



wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

In this complex the dye represented by D in the formula, is bound either directly to a nucleotide or nucleoside base, as used in the foregoing DNA sequencing method, or indirectly to a nucleotide or nucleoside base via a linker. The linker (L) is a linear, branched, cyclic or heterocyclic, saturated or unsaturated group, each having between 1-20 carbon atoms. In this formula, R-O-M_s-B is a natural or modified nucleotide or nucleoside inclusive of DNA chain terminators wherein R can be H, PO_3^{2-}, $P_2O_6^{3-}$, $P_3O_9^{4-}$, alpha-thiophosphates, alpha-BH_3-phosphates and derivatives thereof. O is an oxygen molecule and M_s is a sugar residue, most notably ribose. M_s includes, but is not limited to, ribosyl, 2'-deoxyribosyl, 3'-deoxyribosyl, or 2', 3'-didexyribosyl or 2'-oxabutylribosyl, 2'-oxamethylribosyl as well as other modified ribosesugars. B is representative of a nitrogen-containing heterocyclic base which covers any number of modified bases inclusive of, but not limited to, uracil, thymine, cytosine, guanine, 7-deazaguanine, hypoxanthine, 7-deazahypoxanthine, adenine, or 7-diazaadenine, as well as 2,6-diaminopurine. As discussed earlier, the method of labeling a nucleic acid molecule such as DNA or RNA by incorporating this complex into a nucleic acid chain is one aspect of the present invention. The further method made up of the step of determining the nucleic acid sequence of a molecule based on the information provided by the incorporated complex or complexes, is a further aspect of the present invention.

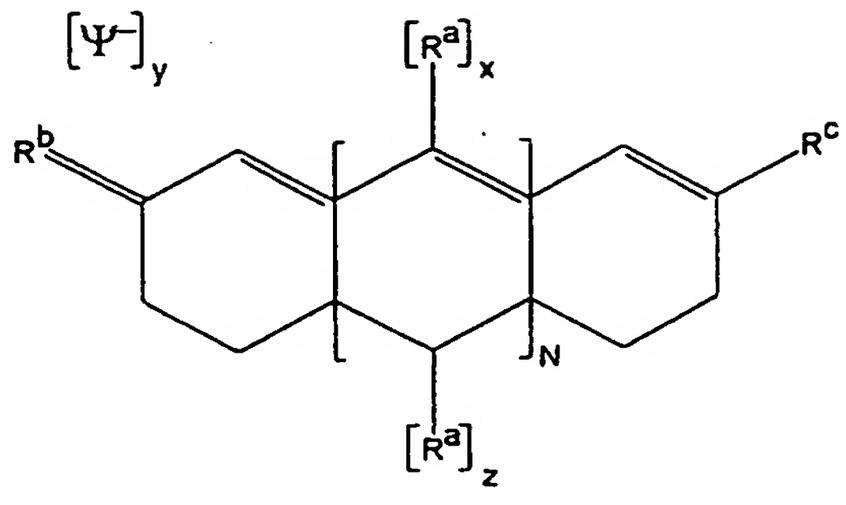
A labeling kit is further provided by the present invention. The kit would provide at least one dye compound of the following formula:



wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

As already exemplified, many types of biological molecules may be chemically labeled with one or more of these dyes including, but not limited to, polysaccharides, nucleic acid polymers such as DNA, RNA, oligonucleotides and oligonucleosides, nucleic acid bases, proteins, cells and lipids. Depending on the group on the biological molecule available for formation of a bond with the reactive substituent on the dye molecule, as well as the nature of the reactive substituent, buffers suitable for chemical labeling of the biological molecule may vary. For example, wherein the reactive moiety on the dye is an isothiocyanato moiety to form a thiourea linkage between the dye label and a primary amine of the biological molecule, a high pH buffer (pH 9.2) such as carbonate buffer may be utilized for labeling purposes. Wherein the dye is of an insoluble nature in aqueous solutions, the dye should contain polar moieties for increasing the solubility of the dye in aqueous solutions. The dye may be included in a lyophilized form to maintain its long term stability upon storage.

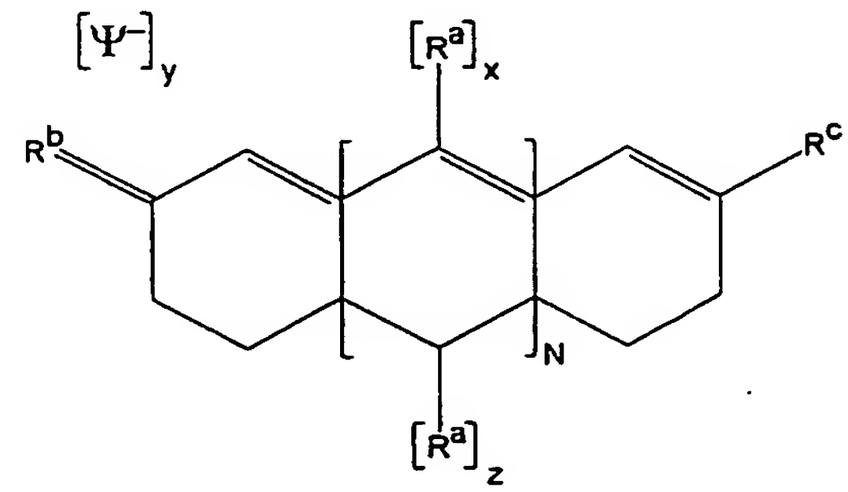
In yet another embodiment of the invention, a DNA sequencing kit is provided which includes a mixture containing dideoxynucleotide chain terminators for use in DNA sequencing methods based on the Sanger method. In this mixture each of the 4 dideoxynucleotide chain terminators corresponding to ddATP, ddGTP, ddCTP and ddTTP would each be bound independently to a dye of the formula:



wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

In one desirable embodiment, each of the terminators would be bound to a dye which has an emission wavelength which is different from that of the other terminators in the mixture.

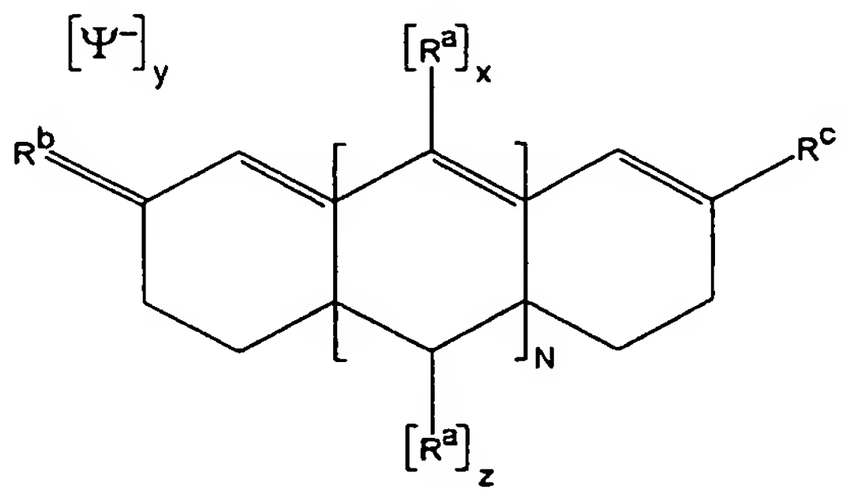
Another DNA sequencing kit is provided for by the present invention having additional components. These components include the same mixture just described containing dideoxynucleotide triphosphate chain terminators, wherein the terminators are each independently bound to a dye of the formula:



wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

Moreover, an oligonucleotide primer complimentary in sequence to a region of a vector template suitable for DNA sequencing, or to a region of the nucleic acid to be copied, a vector suitable for DNA sequencing, a heat-stable DNA polymerase such as Taq polymerase, a standard PCR buffer, and unlabeled deoxynucleotide triphosphates are also included. In a preferred embodiment of the kit, each terminator provided in the mixture has bound to it a dye with an emission wavelength which is different from that of the other terminators in the mixture.

In another embodiment of the present invention, a DNA sequencing kit is provided for wherein the components include an oligonucleotide primer bound to a dye of the formula:



wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

Additional components of this kit include deoxynucleotide triphosphates as well as dideoxynucleotide triphosphate chain terminators, a PCR buffer, a heat-stable DNA polymerase such as Taq polymerase and a vector suitable for DNA sequencing. Here again the Sanger method of DNA sequencing provides for a display of a continuous set of DNA fragments that differs in length by only one nucleotide and relies on random termination of reactions using dideoxynucleotide triphosphate chain terminators which are, in this kit, unlabeled. Reaction mixtures are set up to contain four deoxynucleotide triphosphates and a single dideoxynucleotide triphosphate. The product of four separate reaction mixtures, each containing a different dideoxynucleotide triphosphate, are analyzed. By random termination, reaction 1 using ddATP, containing all A terminations; reaction 2 using ddCTP, contains all

C terminations; and so on. After incubation the reaction products are separated by electrophoresis on polyacrylamide gels and detected as a result of the dye bound to the extended primer via excitation of the flurophore and detection of the infrared emission. The dye-bound oligonucleotide primer provided in the kit can be one which is complementary in sequence to a region of a vector suitable for DNA sequencing or to a region of the nucleic acid to be copied.

In another aspect of the invention there is provided a method of making a partially reduced linear polyarene compound useful as a dye precursor comprising the steps of:

(i) providing an aryl alkali metal compound having alkoxy and carboxylate moieties;

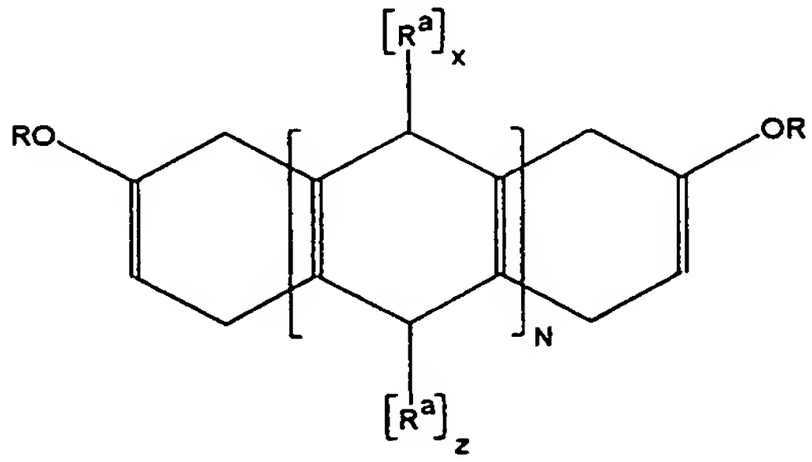
(ii) reacting said compound with an alkoxy aryl acid halide to form a keto-acid;

(iii) reacting said keto-acid under conditions sufficient to produce a closed-ring quinone structure;

(iv) reducing said resultant quinone structure to form a linear polyarene; and

(v) optionally functionalizing said linear polyarene at position R^a; and

(vi) performing a dissolving metal reduction step to produce a compound corresponding to the structure:



Wherein R is methyl or alkyl C_{1-6} and R^a optionally contains a reactive functional group for bonding to a biological molecule, x is 0 or 1 and z is 0 or 1; and N is 1-4.

In one desired aspect of the invention, the aryl alkali metal compound is lithium-2-lithio-5-methoxybenzoate or lithium 3-lithio-7-methoxy-2-naphthoate. Other alkali metals are also useful. The alkoxy aryl acid halide may be selected from a wide variety of materials.

Included among these are *p*-methoxybenzoylchloride, 6-methoxy-2-naphthoylchloride, isophthaloyldichloride and naphthalene-2,7-dicarbonyl chloride.

The keto-acid formed is desirably one of the following: 2-(-4-methoxybenzoyl)-3-methoxybenzoic acid, 2-(6-methoxy-2-naphthoyl)-3-methoxybenzoic acid, 1,3-*bis*-(2-carboxy-4-methoxybenzoyl)-benzene or 2,7-*bis*-(2-carboxy-4-methoxybenzoyl)-naphthalene.

The quinone formed is desirably 2,7-dimethoxyanthraquinone, 2,9-dimethoxy-5,12-naphthacenedione, 2,10-dimethoxy-6,13-pentacenedione, 2,10-dimethoxy-5,7,12,14-pentacenetetraone or 2,11-dimethoxy-5,8,13,16-hexacenetetraone.

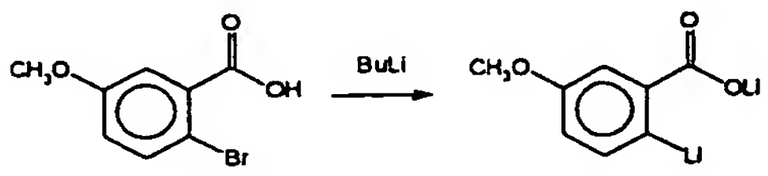
The linear polyarene of step (iv) is desirably 2,7-dimethoxyanthracene, 2,9-dimethoxynaphthacene, 2,10-dimethoxypentacene or 2,11-dimethoxyhexacene.

The partially reduced linear polyarene compound is desirably 2,7-dimethoxy-1,4,5,8,9,10-hexahydroanthracene, 2,9-dimethoxy-1,4,5,6,7,10,11,12-octahydronaphthacene, 2,10-dimethoxy-1,4,5,6,7,8,11,12,13,14-decahydropentacene, or 2,11-dimethoxy-1,4,5,6,7,8,9,12,13,14,15,16-dodecahydrohexacene.

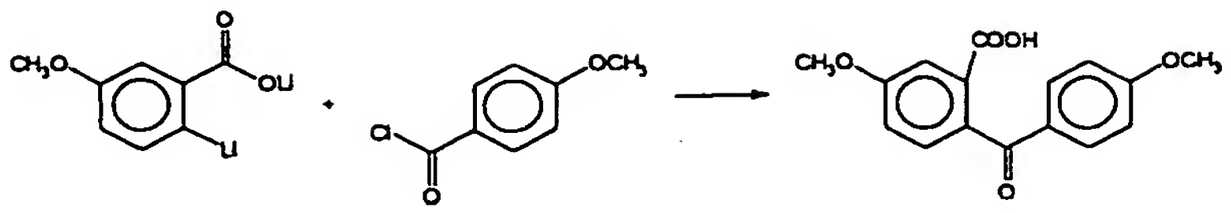
The method further comprises the step of attaching the precursor dye to a biomolecule as previously described herein.

EXAMPLE 1

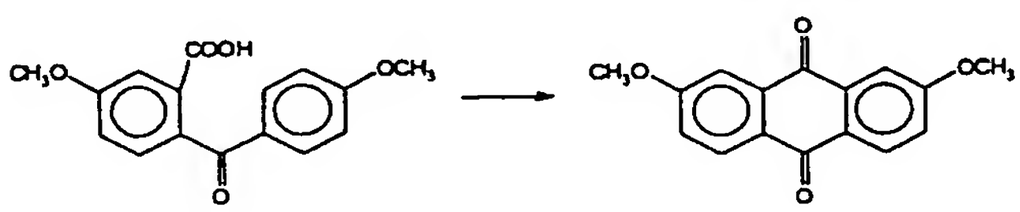
Synthesis of A Dye Precursor With Three Central-Planar Rings



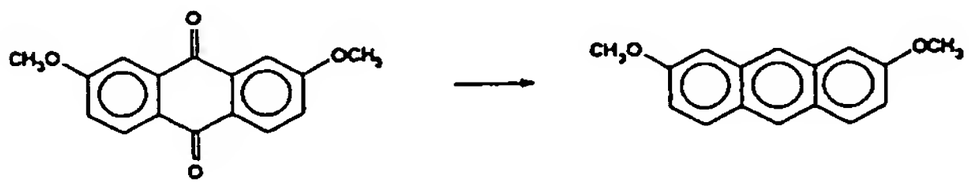
n-Butyllithium (12.5 ml of a 2M solution in hexane; 0.025 mol) is slowly added over one hour to a solution of 5-methoxy-2-bromobenzoic acid (0.0125 mol) in 50 ml dry distilled tetrahydrofuran to form lithium-2-lithio-5-methoxybenzoate. The mixture is maintained under nitrogen or argon atmosphere and the temperature kept at –90 to –100 °C by a liquid nitrogen-diethyl ether bath.



One equivalent of the acid chloride (in this example *p*-methoxybenzoylchloride) dissolved in 25 ml dry tetrahydrofuran is added dropwise to the lithium 5-methoxy-2-lithiobenzoate solution at such a rate that the temperature is kept below –90°C. After stirring at this temperature for one hour, the reaction mixture is poured into 100 ml of 5% hydrochloric acid and stirred for 30 minutes. The layers are separated and the aqueous layer is extracted with ether. The ether layers are combined, washed with water, and are extracted with 10% sodium hydroxide solution. The alkaline extract is then acidified, extracted with ether, dried over sodium sulfate, filtered and concentrated. The residue is purified by recrystallization from benzene-petroleum ether, from chloroform-hexane, or by column chromatography to yield 2-(4-methoxybenzoyl)-3-methoxybenzoic acid..

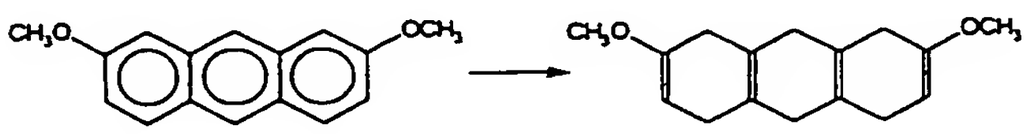


A one gram sample of 2-(4-methoxybenzoyl)-3-methoxybenzoic acid is cooled in ice. Six ml of concentrated sulfuric acid is slowly added with stirring. The resulting solution is allowed to stand for four hours, then it is poured on ice. The resultant solid, 2,7-dimethoxyanthraquinone is recrystallized from acetone-ethanol, from chloroform, or purified via alumina/chloroform column chromatography.



Working under a nitrogen atmosphere, 10 grams of 2,7-dimethoxyanthraquinone were dissolved into 300 ml of a 10% ammonium hydroxide solution and heated to 80°C. After one hour, 75 grams of zinc dust were carefully added in small portions. The temperature was raised to 90°-95°C and allowed to stir at this temperature overnight. After cooling, the solid product and zinc were filter-washed with water until neutral, then extracted under inert

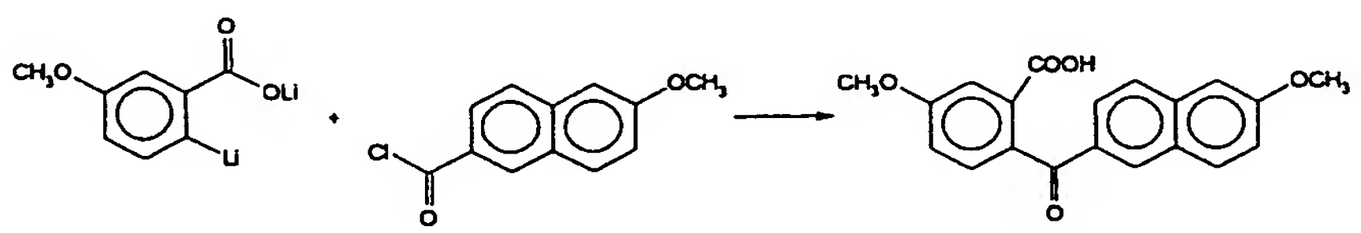
atmosphere with dry methanol for 3 to 4 days using a sohxlet apparatus. After removal of the methanol, the solid extract was purified using column filtration over alumina with chloroform as eluent to yield 2,7-Dimethoxyanthracene. 80% yield, melting point 227-8°C.



Birch Reduction: Under an inert atmosphere were condensed 750 ml liquid ammonia at –40°C. To this solution, 4 g lithium metal were added in small pieces. After the lithium was dissolved, 17.5 g of 2,7-dimethoxyanthracene dissolved in 500 ml dry tetrahydrofuran was added dropwise over one hour. After an additional one hour of stirring at –40°C, the temperature was lowered to about –60°C and 75 ml dry ethanol followed by an additional 4 grams of lithium metal were added, being careful to maintain the temperature at –60°C. After stirring three additional hours, 75 gram of ammonium chloride was added to the reaction and the temperature was allowed to reach room temperature, during which time the ammonia evaporated. The product, 2,7-Dimethoxy-1,4,5,8,9,10-hexahydroanthracene, was precipitated with cold water (0°C), dried in vacuo and recrystallized from hexane. 64% yield, melting point 130-132°C.

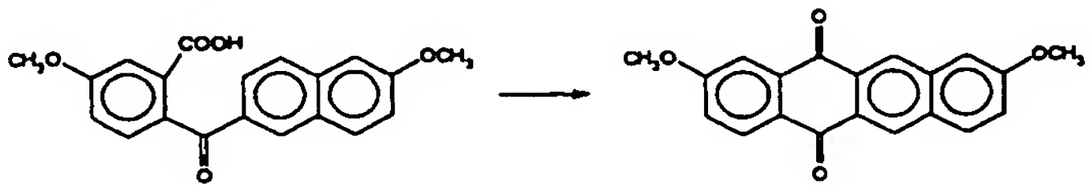
EXAMPLE 2

Synthesis of A Dye Precursor With Four Central-Planar Rings

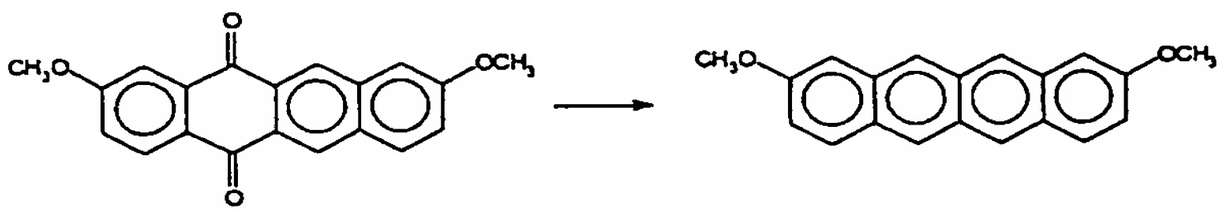


One equivalent of the acid chloride (in this example 6-methoxy-2-napthoylchloride) dissolved in 25 ml dry tetrahydrofuran is added dropwise to the Lithium 5-methoxy-2-lithiobenzoate solution at such a rate that the temperature is kept below –90°C. After stirring at this temperature for one hour, the reaction mixture is poured into 100 ml of 5% hydrochloric acid and stirred for 30 minutes. The layers are separated and the aqueous layer is extracted with ether. The ether layers are combined, washed with water, and are extracted with 10% sodium hydroxide solution. The alkaline extract is then acidified, extracted with

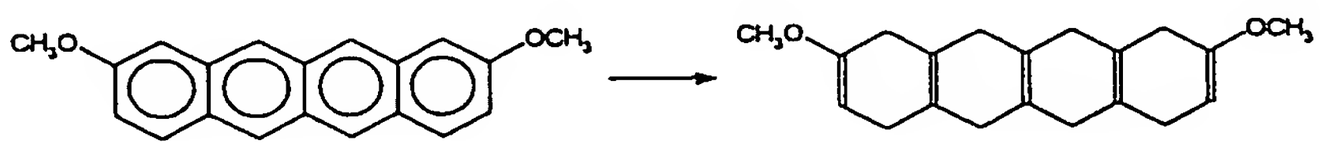
ether, dried over sodium sulfate, filtered and concentrated. The residue is purified by recrystallization from benzene-petroleum ether, from chloroform-hexane, or by column chromatography to yield 2-(6-methoxy-2-naphthoyl)-3-methoxybenzoic acid .



A one gram sample of 2-(6-methoxy-2-naphthoyl)-3-methoxybenzoic acid is cooled in ice. Six ml of concentrated sulfuric acid is slowly added with stirring. The resulting solution is allowed to stand for four hours, then it is poured on ice. The solid is recrystallized from acetone-ethanol, from chloroform, or purified via alumina/chloroform column chromatography to yield 2,9-dimethoxy-5,12-naphthacenedione.



Working under a nitrogen atmosphere, 10 grams of 2,9-dimethoxy-5,12-naphthacenedione are dissolved into 300 ml of a 10% ammonium hydroxide solution and heated to 80°C. After one hour, 70 grams of zinc dust are carefully added in small portions. The temperature is raised to 90°-95°C and allowed to stir at this temperature overnight. After cooling, the solid product and zinc are filter-washed with water until neutral, then extracted under inert atmosphere with dry methanol for 3 to 4 days using a sohxlet apparatus. After removal of the methanol, the solid extract is recrystallized from chloroform-hexane or purified using column filtration over alumina with chloroform as eluent to yield 2,9-dimethoxynaphthacene.



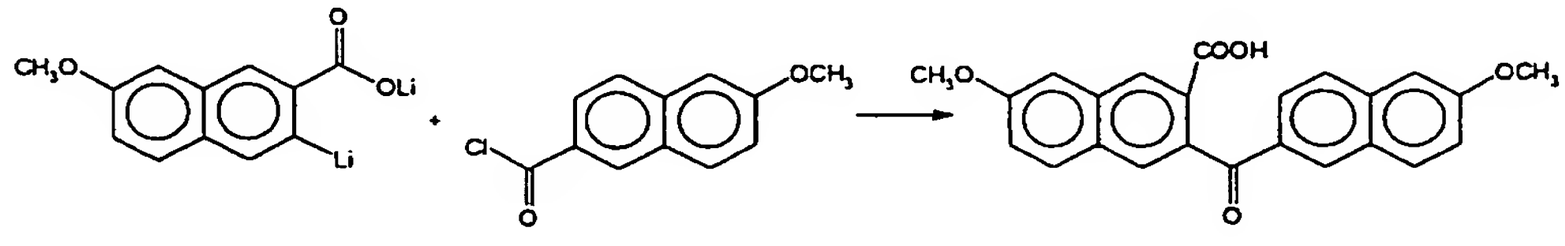
Birch Reduction: Under an inert atmosphere are condensed 800 ml liquid ammonia at –40°C. To this solution, 5 g lithium metal are added in small pieces. After the lithium is dissolved, 17 g of 2,9-dimethoxynaphthacene dissolved in 500 ml dry tetrahydrofuran are added dropwise over one hour. After an additional one hour of stirring at –40°C, the temperature is

lowered to about –60°C and 80 ml dry ethanol followed by an additional 5 grams of lithium metal are added, being careful to maintain the temperature at –60°C. After stirring three additional hours, 80 grams of ammonium chloride are added to the reaction and the temperature is allowed to reach room temperature, during which time the ammonia evaporates. The product, 2,9-dimethoxy-1,4,5,6,7,10,11,12-octahydronaphthacene, is precipitated with cold water (0°C), dried in vacuo and recrystallized from hexane.

EXAMPLE 3

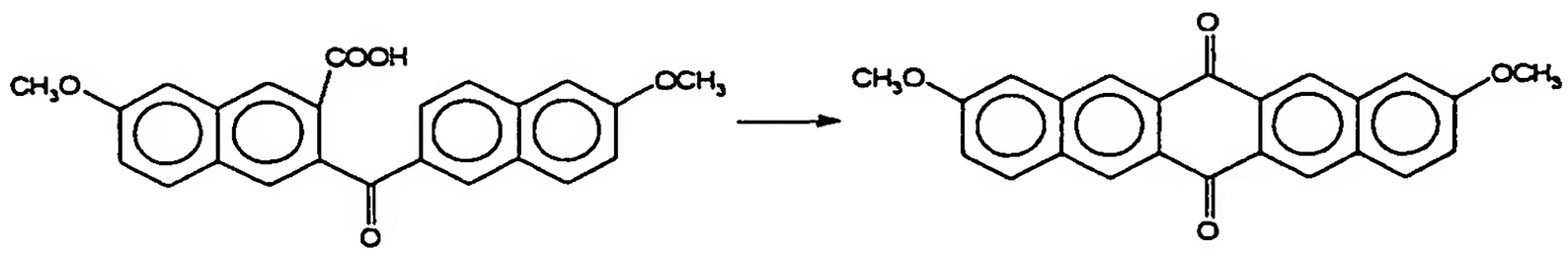
Synthesis of A Dye Precursor With Five Central-Planar Rings (Method A)

n-Butyllithium (12.5 ml of a 2M solution in hexane; 0.025 mol) is slowly added over one hour to a solution of 7-methoxy-3-bromonaphthoic acid (0.0125 mol) in 50 ml dry distilled tetrahydrofuran. The mixture is maintained under nitrogen or argon atmosphere and the temperature kept at –90 to –100 °C by a liquid nitrogen-diethyl ether bath to yield lithium 3-lithio-7-methoxy-2-naphthoate.

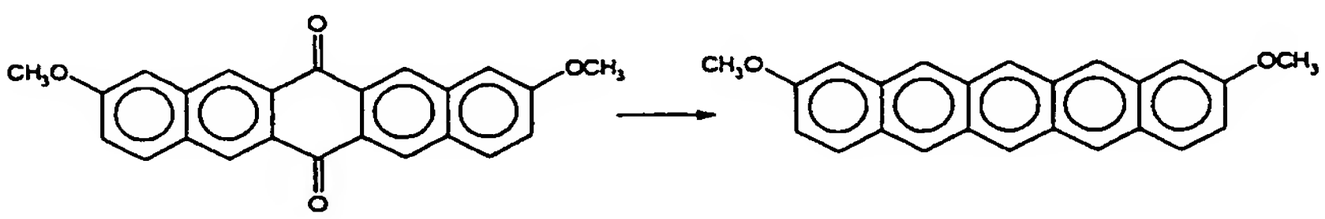


One equivalent of the acid chloride (in this example 6-methoxy-2-napthoylchloride) dissolved in 25 ml dry tetrahydrofuran is added dropwise to the lithium 3-lithio-7-methoxy-2-naphthoate solution at such a rate that the temperature is kept below –90°C. After stirring at this temperature for one hour, the reaction mixture is poured into 100 ml of 5% hydrochloric acid and stirred for 30 minutes. The layers are separated and the aqueous layer is extracted with ether. The ether layers are combined, washed with water, and are extracted with 10% sodium hydroxide solution. The alkaline extract is then acidified, extracted with ether, dried over sodium sulfate, filtered and concentrated. The residue is purified by recrystallization

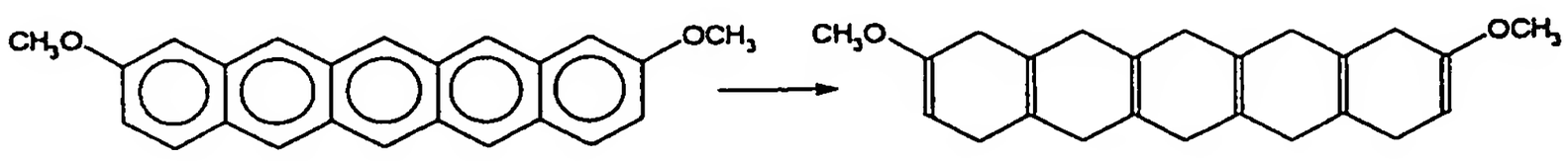
from benzene-petroleum ether, from chloroform-hexane, or by column chromatography to yield 3-(6-methoxy-2-naphthoyl)-7-methoxy-2-naphthoic acid .



A one gram sample of 3-(6-methoxy-2-naphthoyl)-7-methoxy-2-naphthoic acid is cooled in ice. Six ml of concentrated sulfuric acid is slowly added with stirring. The resulting solution is allowed to stand for four hours, then it is poured on ice. The solid is recrystallized from acetone-ethanol, from chloroform, or purified via alumina/chloroform column chromatography to yield 2,10-dimethoxy-6,13-pentacenedione.



Working under a nitrogen atmosphere, 10 grams of 2,10-dimethoxy-6,13-pentacenedione are dissolved into 300 ml of a 10% ammonium hydroxide solution and heated to 80°C. After one hour, 65 grams of zinc dust are carefully added in small portions. The temperature is raised to 90°-95°C and allowed to stir at this temperature overnight. After cooling, the solid product and zinc are filter-washed with water until neutral, then extracted under inert atmosphere with dry methanol for 3 to 4 days using a sohxlet apparatus. After removal of the methanol, the solid extract is recrystallized from chloroform-hexane or purified using column filtration over alumina with chloroform as eluent to yield 2,10-dimethoxypentacene.

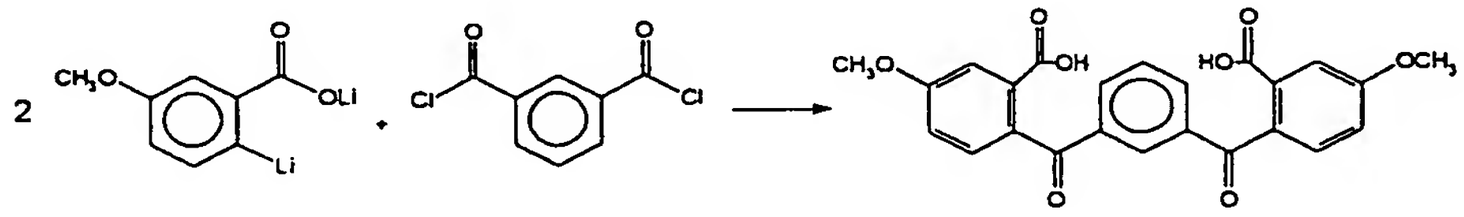


Birch Reduction: Under an inert atmosphere are condensed 850 ml liquid ammonia at –40°C. To this solution, 6 g lithium metal are added in small pieces. After the lithium is dissolved, 17 g of 2,9-dimethoxynaphthacene dissolved in 500 ml dry tetrahydrofuran are added dropwise over one hour. After an additional one hour of stirring at –40°C, the temperature is

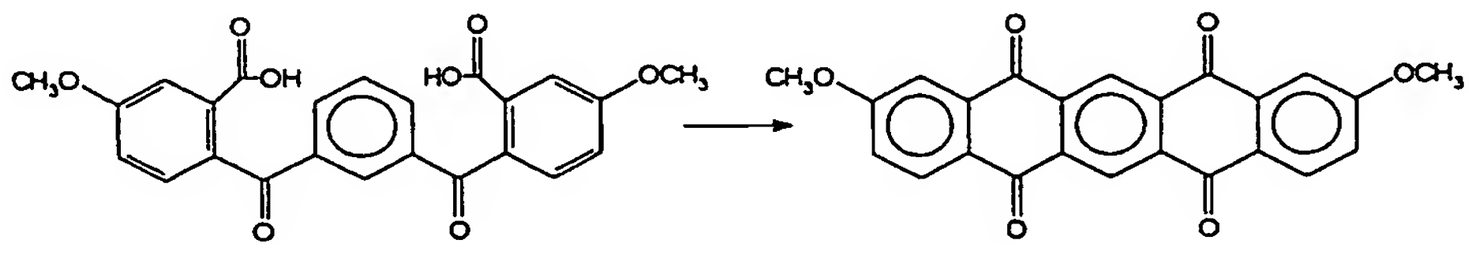
lowered to about –60°C and 80 ml dry ethanol followed by an additional 6 grams of lithium metal are added, being careful to maintain the temperature at –60°C. After stirring three additional hours, 85 grams of ammonium chloride are added to the reaction and the temperature is allowed to reach room temperature, during which time the ammonia evaporates. The product, 2,10-dimethoxy-1,4,5,6,7,8,11,12,13,14-decahydropentacene, is precipitated with cold water (0°C), dried in vacuo and recrystallized from hexane.

EXAMPLE 4

Synthesis of A Dye Precursor With Five Central-Planar Rings (Method B)

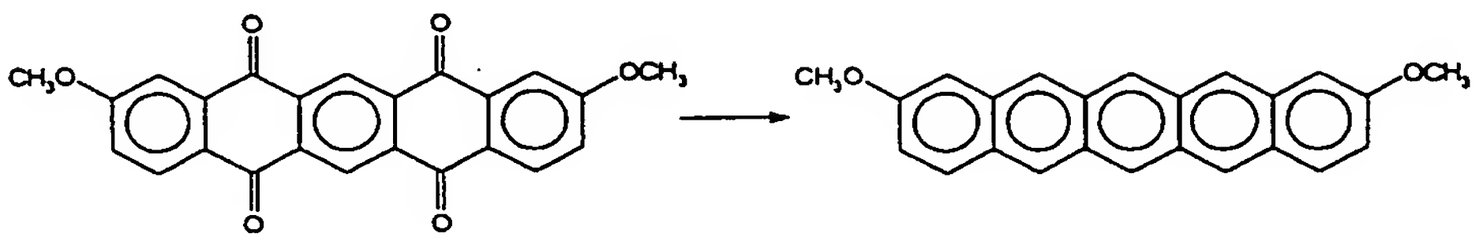


One equivalent of the acid chloride (in this example isophthaloyldichloride) dissolved in 25 ml dry tetrahydrofuran is added dropwise to two equivalents of a lithium 5-methoxy-2-lithiobenzoate solution at such a rate that the temperature is kept below –90°C. After stirring at this temperature for one hour, the reaction mixture is poured into 100 ml of 5% hydrochloric acid and stirred for 30 minutes. The layers are separated and the aqueous layer is extracted with ether. The ether layers are combined, washed with water, and are extracted with 10% sodium hydroxide solution. The alkaline extract is then acidified, extracted with ether, dried over sodium sulfate, filtered and concentrated. The residue is purified by recrystallization from benzene-petroleum ether, from chloroform-hexane, or by column chromatography to yield 1,3-*bis*-(2-Carboxy-4-methoxybenzoyl)-benzene.

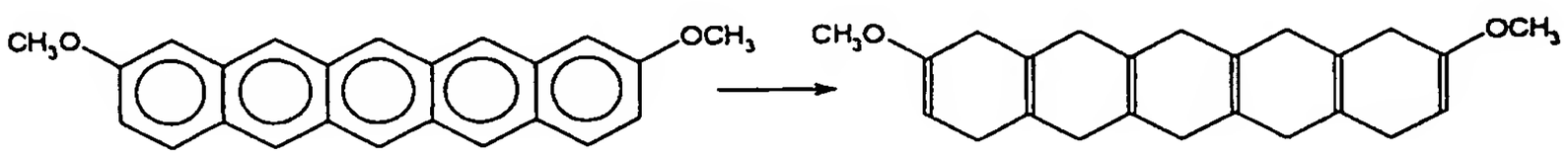


A one gram sample of 1,3-*bis*-(2-Carboxy-4-methoxybenzoyl)-benzene is cooled in ice. Six ml of concentrated sulfuric acid is slowly added with stirring. The resulting solution is allowed to stand for four hours, then it is poured on ice. The solid is recrystallized from

acetone-ethanol, from chloroform, or purified via column chromatography to yield 2,10-dimethoxy-5,7,12,14-pentacenetetraone.



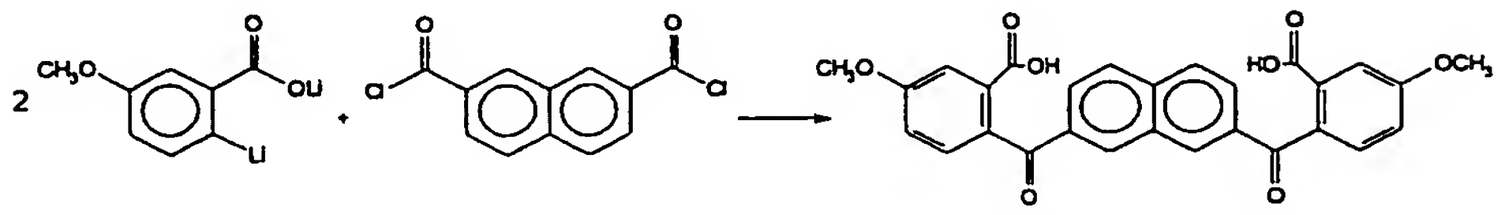
Working under a nitrogen atmosphere, 5 grams of 2,10-dimethoxy-5,7,12,14-pentacenetetraone are dissolved into 300 ml of a 10% ammonium hydroxide solution and heated to 80°C. After one hour, 70 grams of zinc dust are carefully added in small portions. The temperature is raised to 90°-95°C and allowed to stir at this temperature overnight. After cooling, the solid product and zinc are filter-washed with water until neutral, then extracted under inert atmosphere with dry methanol for 3 to 4 days using a sohxlet apparatus. After removal of the methanol, the solid extract is recrystallized from chloroform-hexane or purified using column filtration over alumina with chloroform as eluent to yield 2,10-dimethoxypentacene.



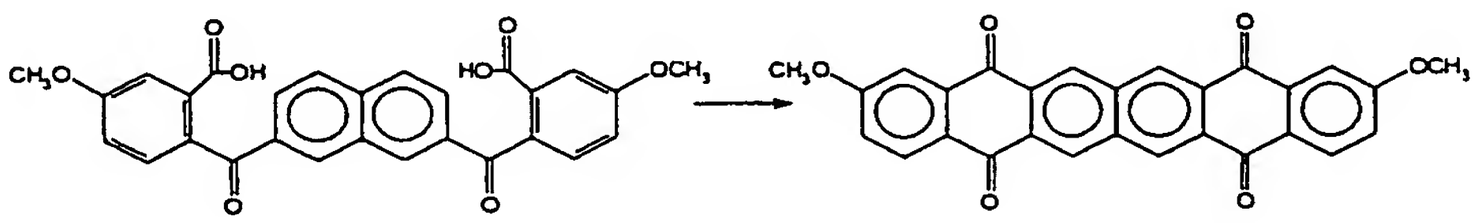
Birch Reduction: Under an inert atmosphere are condensed 850 ml liquid ammonia at –40°C. To this solution, 6 g lithium metal are added in small pieces. After the lithium is dissolved, 17 g of 2,9-dimethoxynaphthacene dissolved in 500 ml dry tetrahydrofuran are added dropwise over one hour. After an additional one hour of stirring at –40°C, the temperature is lowered to about –60°C and 80 ml dry ethanol followed by an additional 6 grams of lithium metal are added, being careful to maintain the temperature at –60°C. After stirring three additional hours, 85 grams of ammonium chloride are added to the reaction and the temperature is allowed to reach room temperature, during which time the ammonia evaporates. The product, 2,10-dimethoxy-1,4,5,6,7,8,11,12,13,14-decahydropentacene, is precipitated with cold water (0°C), dried in vacuo and recrystallized from hexane.

EXAMPLE 5

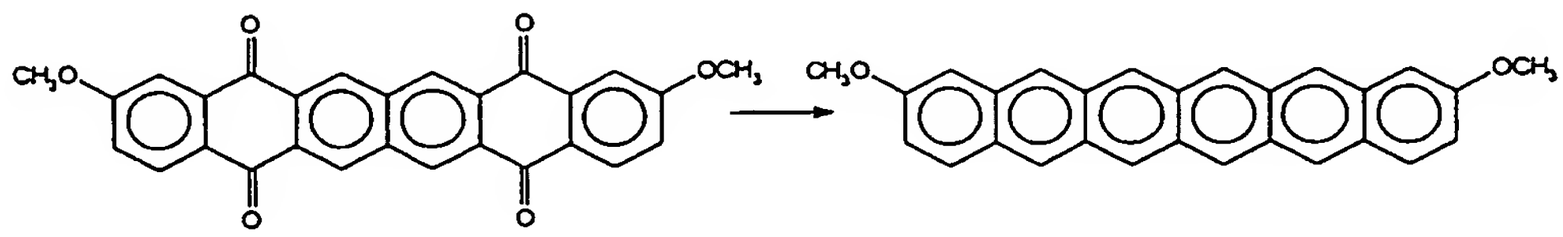
Synthesis of A Dye Precursor With Six Central-Planar Rings



One equivalent of the acid chloride (in this example naphthalene-2,7-dicarbonyl chloride) dissolved in 25 ml dry tetrahydrofuran is added dropwise to two equivalents of a lithium 5-methoxy-2-lithiobenzoate solution at such a rate that the temperature is kept below –90°C. After stirring at this temperature for one hour, the reaction mixture is poured into 100 ml of 5% hydrochloric acid and stirred for 30 minutes. The layers are separated and the aqueous layer is extracted with ether. The ether layers are combined, washed with water, and are extracted with 10% sodium hydroxide solution. The alkaline extract is then acidified, extracted with ether, dried over sodium sulfate, filtered and concentrated. The residue is purified by recrystallization from benzene-petroleum ether, from chloroform-hexane, or by column chromatography to yield 2,7-*bis*-(2-Carboxy-4-methoxybenzoyl)-naphthalene.

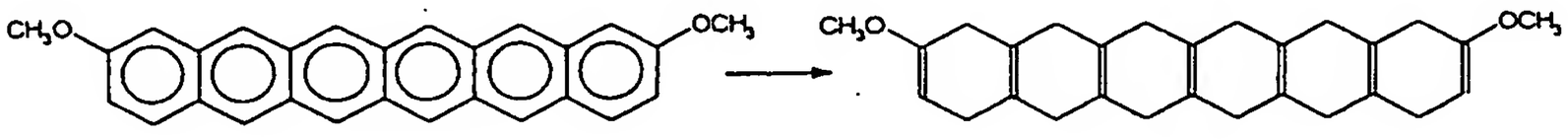


A one gram sample of 2,7-*bis*-(2-Carboxy-4-methoxybenzoyl)-naphthalene is cooled in ice. Six ml of concentrated sulfuric acid is slowly added with stirring. The resulting solution is allowed to stand for four hours, then it is poured on ice. The solid is recrystallized from acetone-ethanol, from chloroform, or purified via column chromatography to yield 2,11-Dimethoxy-5,8,13,16-hexacenetetraone.



Working under a nitrogen atmosphere, 5 grams of 2,11-Dimethoxy-5,8,13,16-hexacenetetraone are dissolved into 300 ml of a 10% ammonium hydroxide solution and

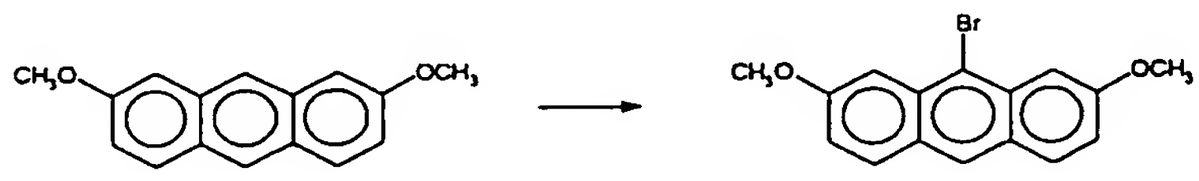
heated to 80°C. After one hour, 65 grams of zinc dust are carefully added in small portions. The temperature is raised to 90°-95°C and allowed to stir at this temperature overnight. After cooling, the solid product and zinc are filter-washed with water until neutral, then extracted under inert atmosphere with dry methanol for 3 to 4 days using a sohxlet apparatus. After removal of the methanol, the solid extract is recrystallized from chloroform-hexane or purified using column filtration over alumina with chloroform as eluent to yield 2,11-dimethoxyhexacene.



Birch Reduction: Under an inert atmosphere are condensed 900 ml liquid ammonia at –40°C. To this solution, 8 g lithium metal are added in small pieces. After the lithium is dissolved, 17 g of 2,11-dimethoxyhexacene dissolved in 500 ml dry tetrahydrofuran are added dropwise over one hour. After an additional one hour of stirring at –40°C, the temperature is lowered to about –60°C and 90 ml dry ethanol followed by an additional 8 grams of lithium metal are added, being careful to maintain the temperature at –60°C. After stirring three additional hours, 90 grams of ammonium chloride are added to the reaction and the temperature is allowed to reach room temperature, during which time the ammonia evaporates. The product, 2,11-dimethoxy-1,4,5,6,7,8,9,12,13,14,15,16-dodecahydrohexacene, is precipitated with cold water (0°C), dried in vacuo and recrystallized from hexane.

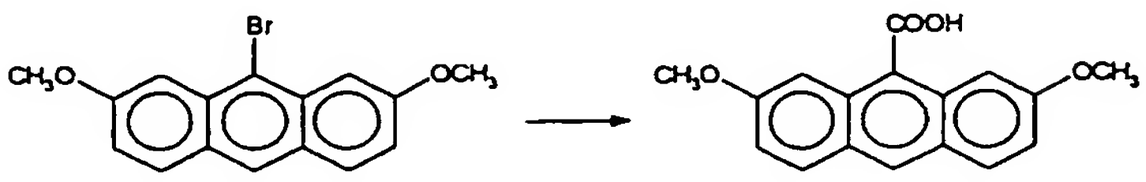
EXAMPLE 6

Placement of a Reactive Carboxylic Acid Group at Position 9 of a 3-Ring Dye Precursor

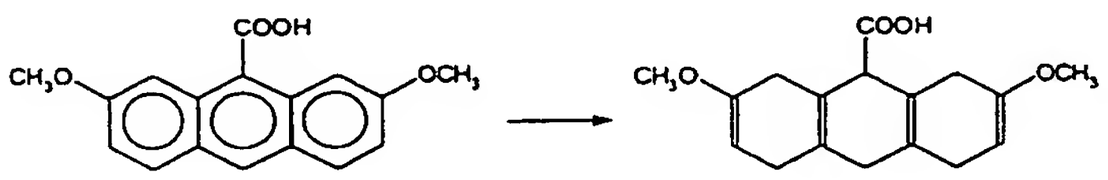


A solution of *N*-bromosuccinimide (1 mmol) in dry, distilled dimethylformamide (5 ml) is added to a solution of 2,7 dimethoxy-anthracene in dry DMF and stirred at room temperature for 24 hours. The mixture is then poured into 50 ml water and extracted with petroleum ether or methylene chloride. The extract is washed with water, dried over sodium sulfate and

evaporated to yield the crude monobromide product, which is purified by recrystallization from chloroform-hexane or by column chromatography.



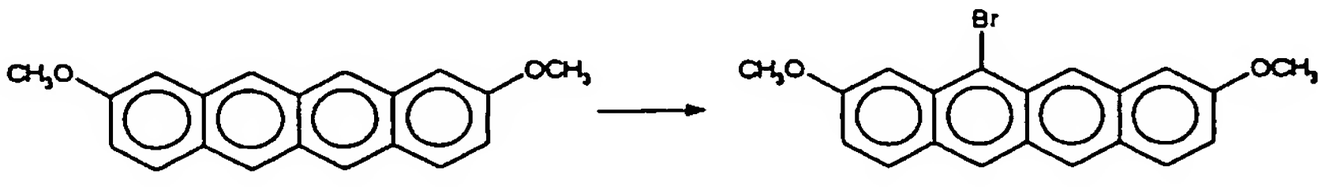
Into 15 ml dry distilled THF are placed 4 mmol magnesium and 0.2 ml dibromoethane. The mixture is stirred and refluxed for one hour and then cooled, after which 0.15 g potassium metal is carefully added. The mixture is carefully reheated with stirring for one hour. A solution of 4 mmol of the bromide in 20 ml dry distilled THF is added via dropping funnel over 30 minutes and the mixture is refluxed for an additional hour. Small pieces of solid carbon dioxide are then added to the reaction (approximately 10 grams). The mixture is refluxed for 30 minutes and the reaction is carefully poured over ice cold 5% hydrochloric acid and stirred for 30 minutes. The layers are separated and the aqueous layer is extracted with ether. The ether layers are combined, washed with water, and are extracted with 10% sodium hydroxide solution. The alkaline extract is then acidified, extracted with ether, dried over sodium sulfate, filtered and concentrated. The residue is purified by recrystallization from benzene-petroleum ether, from chloroform-hexane, or by column chromatography.



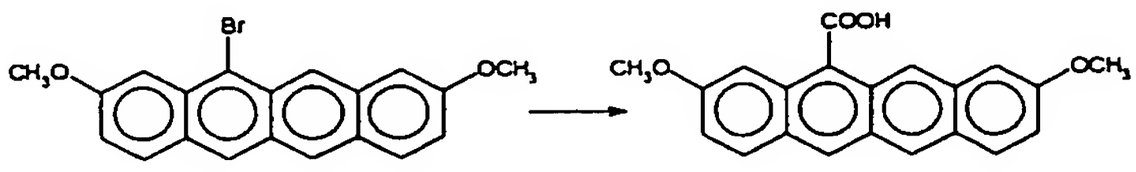
Birch Reduction: Under an inert atmosphere are condensed 750 ml liquid ammonia at –40°C. To this solution, 4 g lithium metal are added in small pieces. After the lithium is dissolved, 70 mmol of 2,7-dimethoxy-9-anthracenecarboxylic acid dissolved in 500 ml dry tetrahydrofuran is added dropwise over one hour. After an additional one hour of stirring at –40°C, the temperature is lowered to about –60°C and 75 ml dry ethanol followed by an additional 4 grams of lithium metal are added, being careful to maintain the temperature at –60°C. After stirring three additional hours, 75 gram of ammonium chloride are added to the reaction and the temperature is allowed to reach room temperature, during which time the ammonia is evaporated. The product is precipitated with cold water (0°C), dried in vacuo and recrystallized from hexane.

EXAMPLE 7

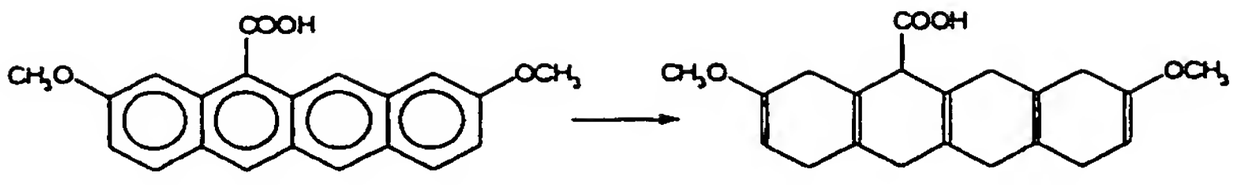
Placement of a Reactive Carboxylic Acid Group at Position 11 of a 4-Ring Dye Precursor



A solution of *N*-bromosuccinimide (1 mmol) in dry, distilled dimethylformamide (5 ml) is added to a solution of 2,9-dimethoxynaphthacene in dry DMF and stirred at room temperature for 24 hours. The mixture is then poured into 50 ml water and extracted with petroleum ether or methylene chloride. The extract is washed with water, dried over sodium sulfate and evaporated to yield the crude monobromide product, which is purified by recrystallization from chloroform-hexane or by column chromatography.



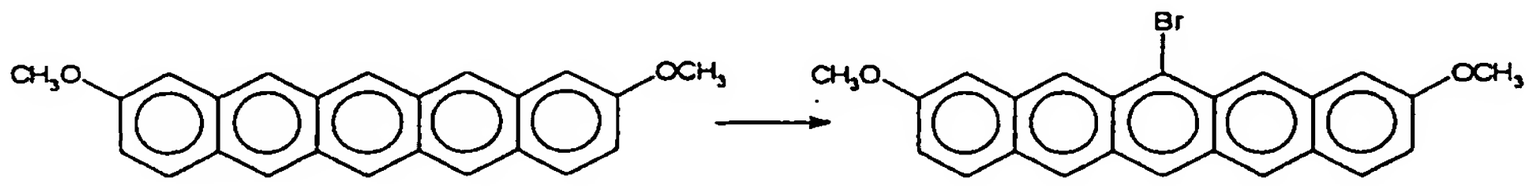
Into 15 ml dry distilled THF are placed 4 mmol magnesium and 0.2 ml dibromoethane. The mixture is stirred and refluxed for one hour and then cooled, after which 0.15 g potassium metal is carefully added. The mixture is carefully reheated with stirring for one hour. A solution of 4 mmol of the bromide in 20 ml dry distilled THF is added via dropping funnel over 30 minutes and the mixture is refluxed for an additional hour. Small pieces of solid carbon dioxide are then added to the reaction (approximately 10 grams). The mixture is refluxed for 30 minutes and the reaction is carefully poured over ice cold 5% hydrochloric acid and stirred for 30 minutes. The layers are separated and the aqueous layer is extracted with ether. The ether layers are combined, washed with water, and are extracted with 10% sodium hydroxide solution. The alkaline extract is then acidified, extracted with ether, dried over sodium sulfate, filtered and concentrated. The residue is purified by recrystallization from benzene-petroleum ether, from chloroform-hexane, or by column chromatography.



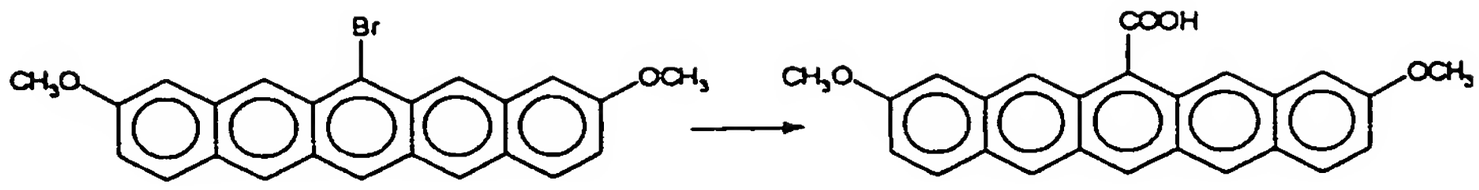
Birch Reduction: Under an inert atmosphere are condensed 800 ml liquid ammonia at –40°C. To this solution, 5 g lithium metal are added in small pieces. After the lithium is dissolved, 70 mmol of 2,9-Dimethoxy-11-naphthacenecarboxylic acid dissolved in 500 ml dry tetrahydrofuran are added dropwise over one hour. After an additional one hour of stirring at –40°C, the temperature is lowered to about –60°C and 80 ml dry ethanol followed by an additional 5 grams of lithium metal are added, being careful to maintain the temperature at –60°C. After stirring three additional hours, 80 grams of ammonium chloride are added to the reaction and the temperature is allowed to reach room temperature, during which time the ammonia evaporates. The product is precipitated with cold water (0°C), dried in vacuo and recrystallized from hexane.

EXAMPLE 8

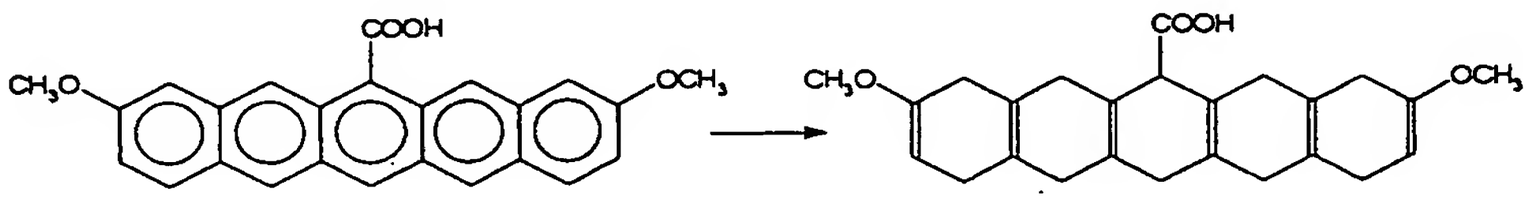
Placement of a Reactive Carboxylic Acid Group at Position 13 of a 5-Ring Dye Precursor



A solution of *N*-bromosuccinimide (1 mmol) in dry, distilled dimethylformamide (5 ml) is added to a solution of 2,10-dimethoxypentacene in dry DMF and stirred at room temperature for 24 hours. The mixture is then poured into 50 ml water and extracted with petroleum ether or methylene chloride. The extract is washed with water, dried over sodium sulfate and evaporated to yield the crude monobromide product, which is purified by recrystallization from chloroform-hexane or by column chromatography.



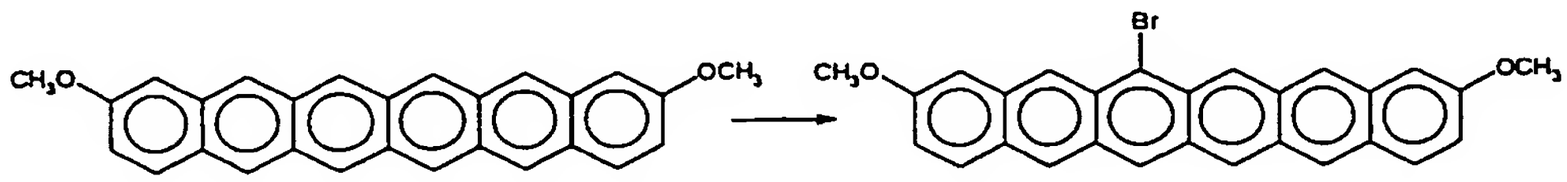
Into 15 ml dry distilled THF are placed 4 mmol magnesium and 0.2 ml dibromoethane. The mixture is stirred and refluxed for one hour and then cooled, after which 0.15 g potassium metal is carefully added. The mixture is carefully reheated with stirring for one hour. A solution of 4 mmol of the bromide in 20 ml dry distilled THF is added via dropping funnel over 30 minutes and the mixture is refluxed for an additional hour. Small pieces of solid carbon dioxide are then added to the reaction (approximately 10 grams). The mixture is refluxed for 30 minutes and the reaction is carefully poured over ice cold 5% hydrochloric acid and stirred for 30 minutes. The layers are separated and the aqueous layer is extracted with ether. The ether layers are combined, washed with water, and are extracted with 10% sodium hydroxide solution. The alkaline extract is then acidified, extracted with ether, dried over sodium sulfate, filtered and concentrated. The residue is purified by recrystallization from benzene-petroleum ether, from chloroform-hexane, or by column chromatography.



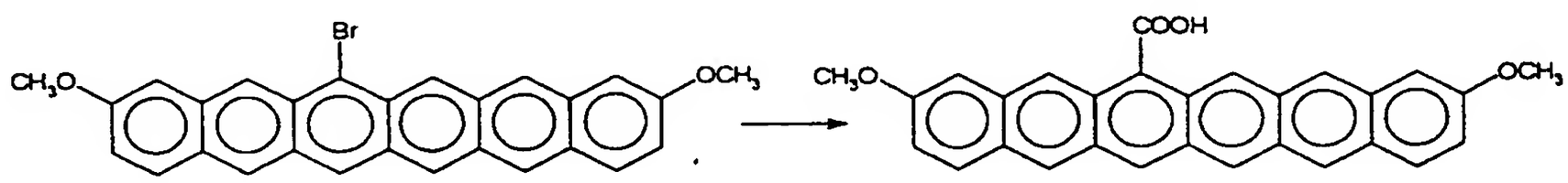
Birch Reduction: Under an inert atmosphere are condensed 850 ml liquid ammonia at –40°C. To this solution, 6 g lithium metal are added in small pieces. After the lithium is dissolved, 70 mmol of 2,10-dimethoxy-13-pentacenecarboxylic acid dissolved in 500 ml dry tetrahydrofuran are added dropwise over one hour. After an additional one hour of stirring at –40°C, the temperature is lowered to about –60°C and 80 ml dry ethanol followed by an additional 6 grams of lithium metal are added, being careful to maintain the temperature at –60°C. After stirring three additional hours, 85 grams of ammonium chloride are added to the reaction and the temperature is allowed to reach room temperature, during which time the ammonia evaporates. The product is precipitated with cold water (0°C), dried in vacuo and recrystallized from hexane.

EXAMPLE 9

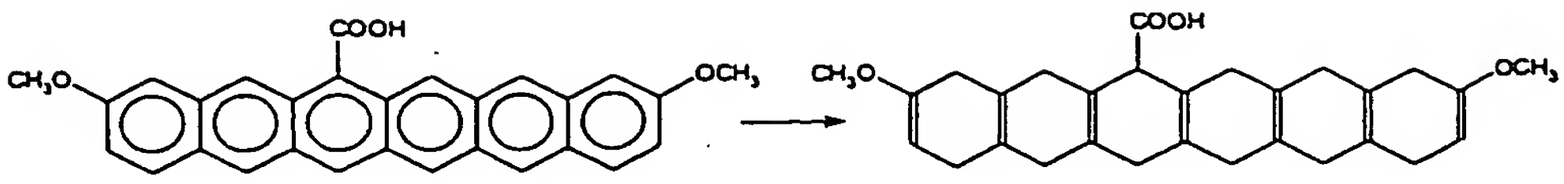
Placement of a Reactive Carboxylic Acid Group at Position 14 of a 6-Ring Dye Precursor



A solution of *N*-bromosuccinimide (1 mmol) in dry, distilled dimethylformamide (5 ml) is added to a solution of 2,11-dimethoxyhexacene in dry DMF and stirred at room temperature for 24 hours. The mixture is then poured into 50 ml water and extracted with petroleum ether or methylene chloride. The extract is washed with water, dried over sodium sulfate and evaporated to yield the crude monobromide product, which is purified by recrystallization from chloroform-hexane or by column chromatography.



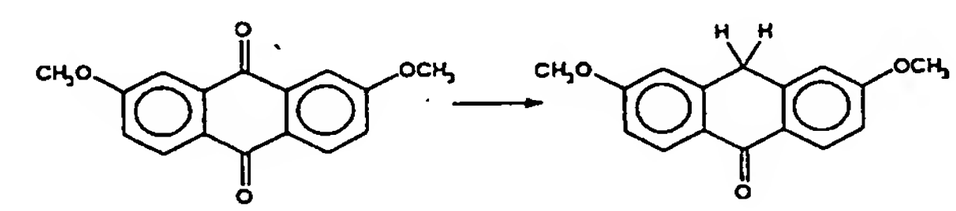
Into 15 ml dry distilled THF are placed 4 mmol magnesium and 0.2 ml dibromoethane. The mixture is stirred and refluxed for one hour and then cooled, after which 0.15 g potassium metal is carefully added. The mixture is carefully reheated with stirring for one hour. A solution of 4 mmol of the bromide in 20 ml dry distilled THF is added via dropping funnel over 30 minutes and the mixture is refluxed for an additional hour. Small pieces of solid carbon dioxide are then added to the reaction (approximately 10 grams). The mixture is refluxed for 30 minutes and the reaction is carefully poured over ice cold 5% hydrochloric acid and stirred for 30 minutes. The layers are separated and the aqueous layer is extracted with ether. The ether layers are combined, washed with water, and are extracted with 10% sodium hydroxide solution. The alkaline extract is then acidified, extracted with ether, dried over sodium sulfate, filtered and concentrated. The residue is purified by recrystallization from benzene-petroleum ether, from chloroform-hexane, or by column chromatography.

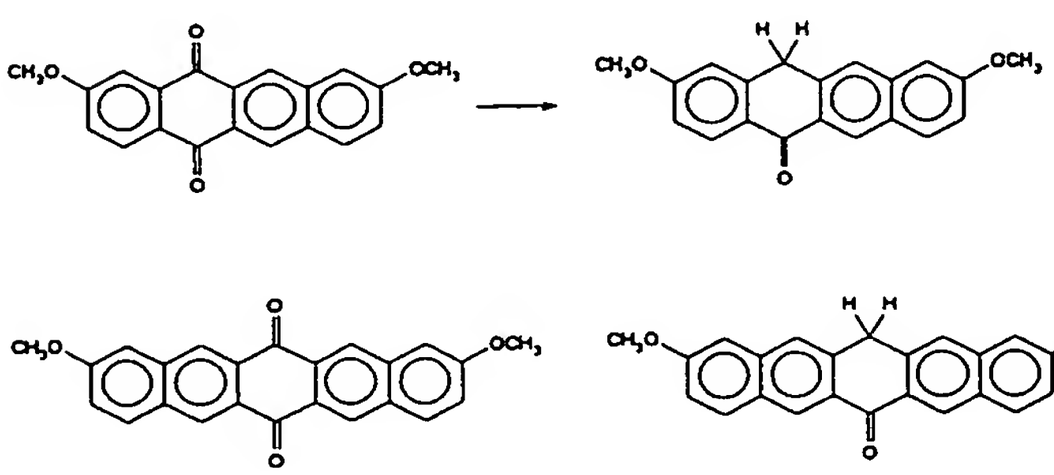


Birch Reduction: Under an inert atmosphere are condensed 900 ml liquid ammonia at –40°C. To this solution, 8 g lithium metal are added in small pieces. After the lithium is dissolved, 70 mmol of 2,11-Dimethoxy-14-hexacenecarboxylic acid dissolved in 500 ml dry tetrahydrofuran are added dropwise over one hour. After an additional one hour of stirring at –40°C, the temperature is lowered to about –60°C and 90 ml dry ethanol followed by an additional 8 grams of lithium metal are added, being careful to maintain the temperature at –60°C. After stirring three additional hours, 90 grams of ammonium chloride are added to the reaction and the temperature is allowed to reach room temperature, during which time the ammonia evaporates. The product is precipitated with cold water (0°C), dried in vacuo and recrystallized from hexane.

EXAMPLE 10

Placement of a Carboxylic Acid Group on 3, 4 and 5-Ring Dye Precursors at Positions 10, 6 and 6, Respectively

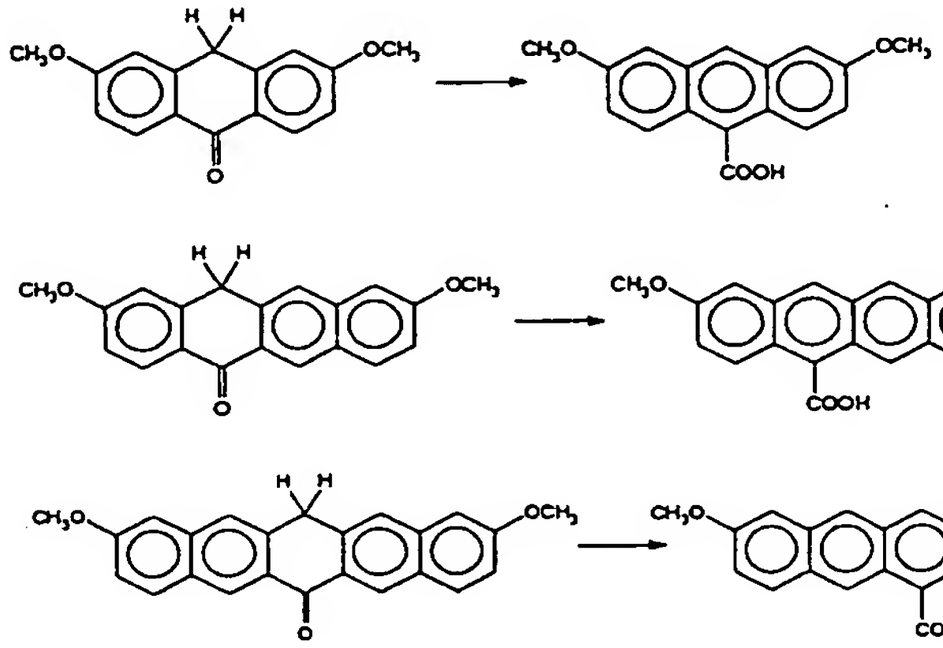




Generalized procedure for formation of anthracene, naphthacene, and pentacene compounds.

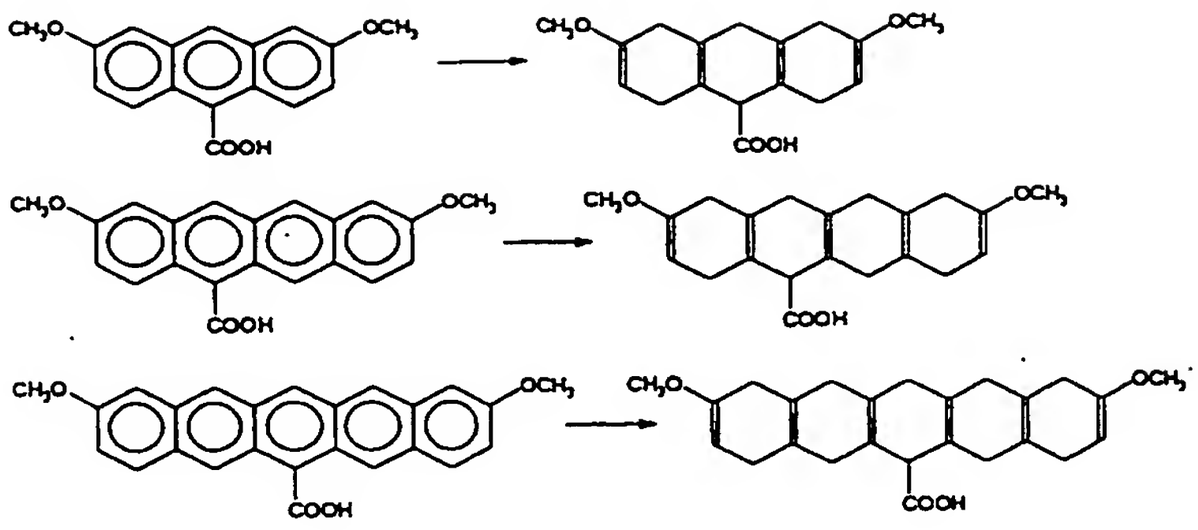
In a one liter flask fitted with a mechanical stirrer, 35 mmol of the quinone is dissolved in 120 ml concentrated sulfuric acid with stirring at 20°C. To the reaction are added 5 ml of

water and 75 mmol copper dust, and the reaction is heated to 40°C until the copper has completely dissolved. The reaction mixture is then poured into 400 ml boiling water and allowed to stand overnight. The precipitate is vacuum filtered, washed neutral with water, dried in vacuo and recrystallized from chloroform-ethanol or purified by column chromatography.



Generalized procedure for formation of dimethoxy-arene carboxylic acids.

Into a 500 ml flask fitted with a mechanical stirrer is placed 100 ml tetrahydrofuran and 20 mmol lithium metal. The reaction mixture is cooled to –40°C using an acetone-dry ice bath, and to the solution is added via dropping funnel a solution of 10 mmol bromomethyl methylether in 10 ml tetrahydrofuran. The reaction is stirred for one hour at –40°C and to the reaction is then added 9 mmol of the ketone. The reaction temperature is maintained for an additional two hours, upon which the reaction is allowed to warm to room temperature. The solvent is removed by vacuum and the adduct is oxidized to the carboxyarene by addition of 15 ml water and 60 mmol bromine at 0°C. The reaction is stirred at room temperature for 3 days. The reaction is carefully poured over ice cold 5% hydrochloric acid and stirred for 30 minutes. The layers are separated and the aqueous layer is extracted with ether. The ether layers are combined, washed with water, and are extracted with 10% sodium hydroxide solution. The alkaline extract is then acidified, extracted with ether, dried over sodium sulfate, filtered and concentrated. The residue is purified by recrystallization from benzene-petroleum ether, from chloroform-hexane, or by column chromatography.

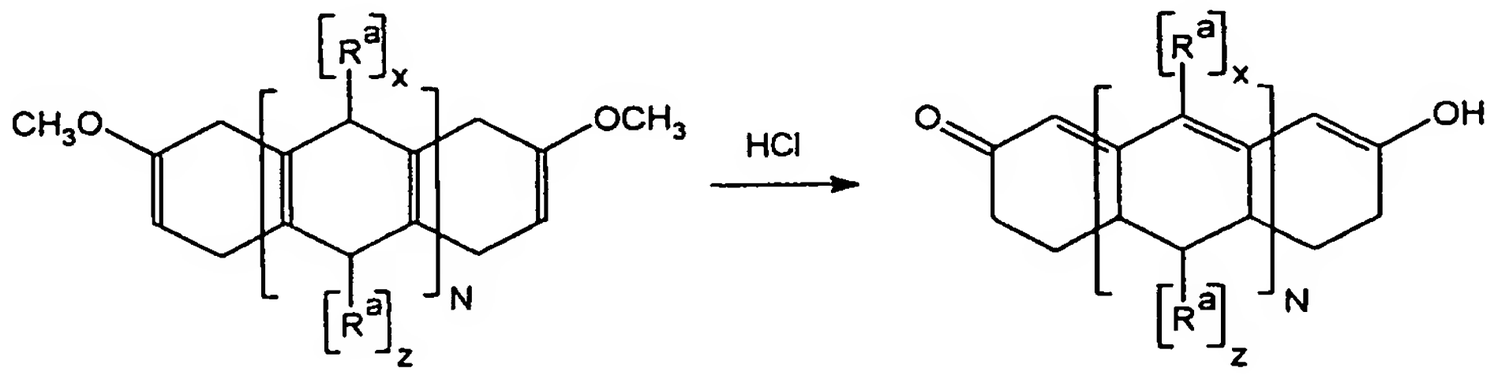


Generalized procedure for Birch reduction of dimethoxy-arene carboxylic acid compounds.

Under an inert atmosphere are condensed 900 ml liquid ammonia at –40°C. To this solution, between 4 and 8 g lithium metal are added in small pieces. After the lithium is dissolved, 70 mmol of the arenecarboxylic acid dissolved in 500 ml dry tetrahydrofuran are added dropwise over one hour. After an additional one hour of stirring at –40°C, the temperature is lowered to about –60°C and 90 ml dry ethanol followed by an additional 4 to 8 grams of lithium metal are added, being careful to maintain the temperature at –60°C. After stirring three additional hours, 90 grams of ammonium chloride are added to the reaction and the temperature is allowed to reach room temperature, during which time the ammonia evaporates. The product is precipitated with cold water (0°C), dried in vacuo and recrystallized from chloroform-hexane.

EXAMPLE 11

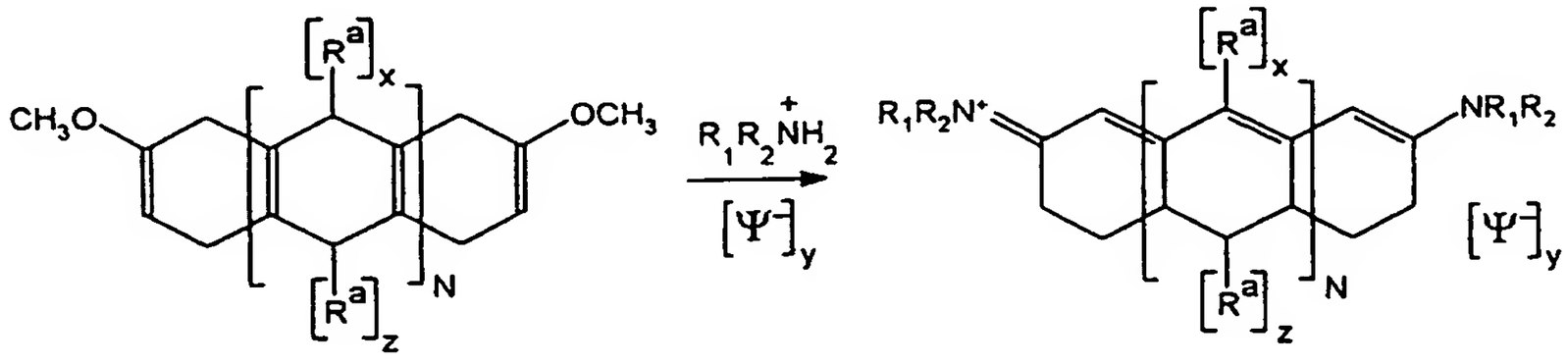
General procedure for the conversion of Birch reduction products to oxo-dyes



5 mmol of the Birch product is suspended in 15 ml 1 M HCl and warmed to 80°C for one hour. After cooling, the product precipitates, washed, and vacuum dried. Purification is accomplished by recrystallization from chloroform-hexane.

EXAMPLE 12

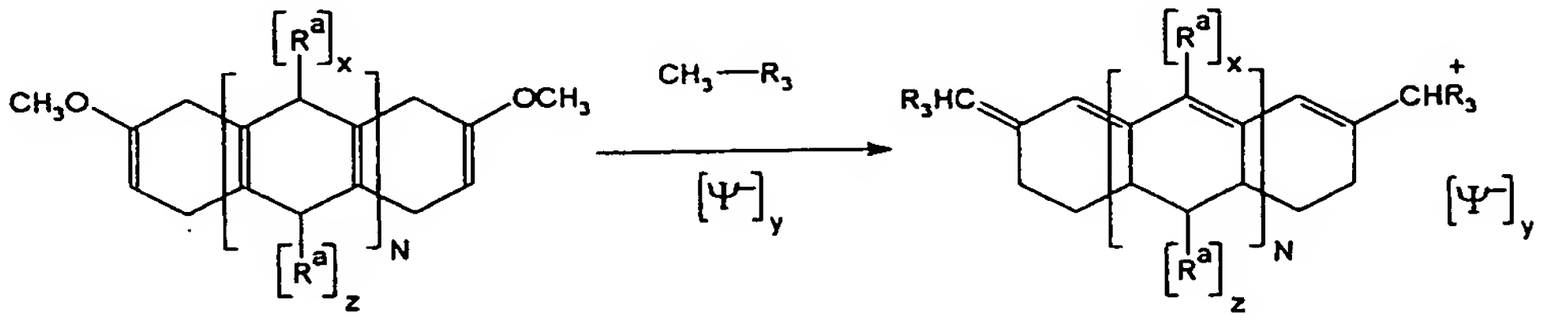
General procedure for the conversion of Birch reduction products to amine-dyes



Into a 100 ml flask are placed 10 mmol of powdered substituted or unsubstituted primary or secondary aromatic amine (i.e. p-nitroanilinium chloride, p-chloroanilinium chloride) and 5 mmol of the Birch reduction product (i.e. 2,7-Dimethoxy-1,4,5,8,9,10-hexahydroanthracene) and the solids are heated to 140-170°C for 10 minutes to melt. 20 mmol triethylamine in 10 ml pyridine are added, heated to reflux for 10 minutes. 10 mmol sodium iodide in 50 ml ethanol are added and refluxed for three minutes. The solution is left standing overnight at 0°C. The solid is filtered, washed, and recrystallized or purified via column chromatography.

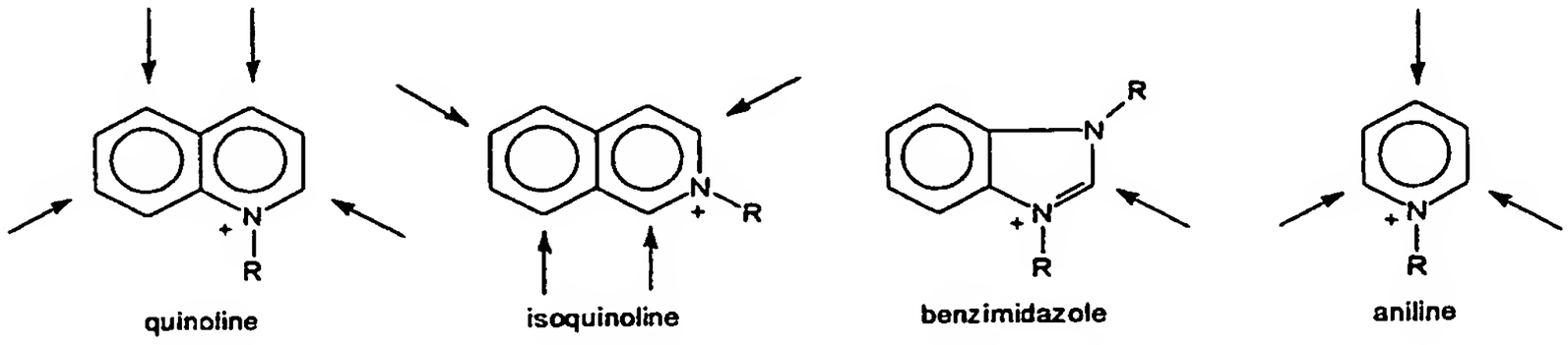
EXAMPLE 13

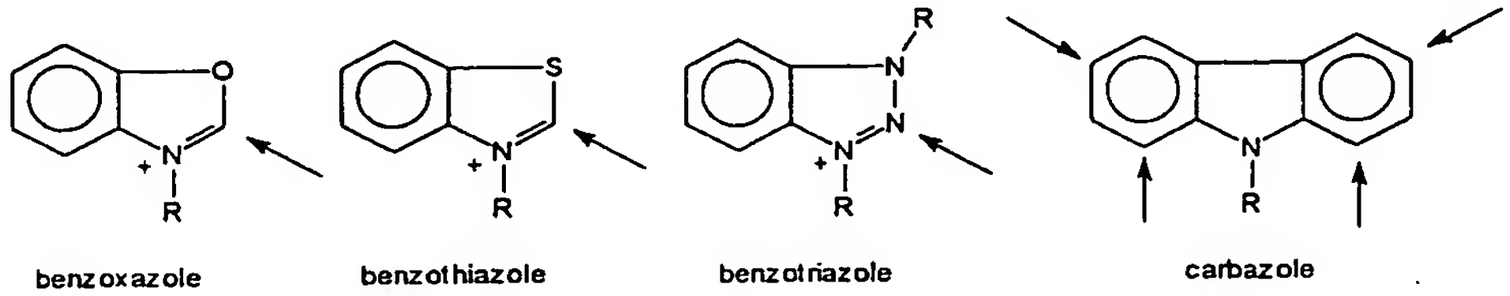
General procedure for the addition of other end groups to Birch reduction products



Into a 100 ml flask are placed 10 mmol of compound, CH_3R_3, (where R_3 is selected from one of the moieties shown below or substituted versions thereof and attached at one of the positions marked with an arrow and where the hydrogens of the methyl group CH_3 are relatively acidic due to the stabilization of the conjugate base and its negative charge by the strategic presence of a nitrogen atom two, four, or six atoms in distance from the methyl group.) 10 mmol toluenesulfonic acid, and 5 mmol of the Birch reduction product (i.e. 2,7-Dimethoxy-1,4,5,8,9,10-hexahydroanthracene) and the solids are heated to 140-170°C for 10 minutes to melt. 20 mmol triethylamine in 10 ml pyridine are added, heated to reflux for 10

minutes. 10 mmol sodium iodide in 50 ml ethanol are added and refluxed for three minutes. The solution is left standing overnight at 0°C. The solid is filtered, washed, and recrystallized or purified via column chromatography.

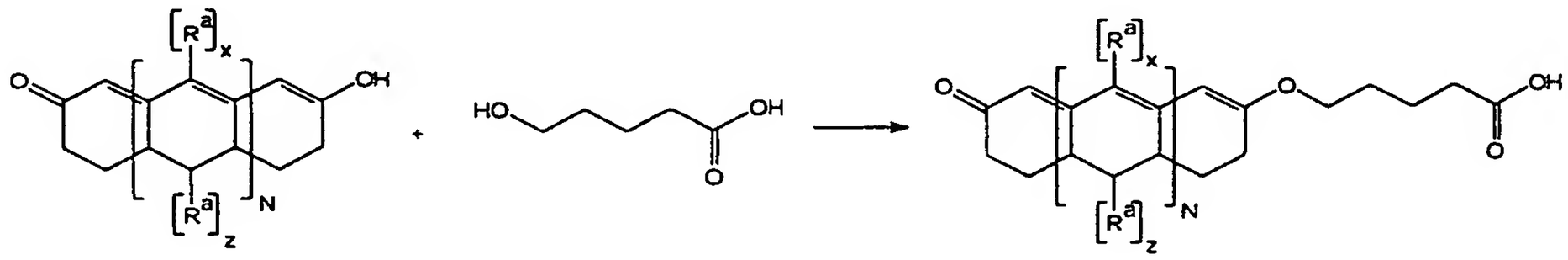




Where R = H, alkyl, alkenyl, alkynyl, aryl or substituted versions thereof which may also contain a reactive but separate substituent selected from phosphoramidite, isothiocyanate, isocyanate, monochlorotriazine, dichlorotriazine, mono-or di-halogen substituted pyridine, mono-or di-halogen substituted diazine, aziridine, sulfonyl halide, acid halide, glyoxal, reactive ester, aldehyde, alcohol, hydroxyl group, carboxylic group, amine, amide, thiol, amino group, sulfonyl halide, azides, acids, hydrazines, ketones, haloacetamides, hydroxysuccinimide ester, hydroxysulfosuccinimide ester and imido ester for covalent attachment to a biological molecule or which may also contain a polar moiety to increase solubility of said dye in aqueous solutions selected from hydroxy, nitro, sulfonate, sulfate, carboxylate, substituted amine, quaternary amine and nitrile.

EXAMPLE 14

General procedure for the covalent attachment of 4-hydroxy butyric acid linker to an oxy-dye



50 mg of the oxo dye in 5 mL chloroform were placed in a 50 mL round bottomed flask and mixed. To the heterogeneous mixture, 0.5 mL concentrated sulfuric acid was carefully added. Two phases were observed. An additional 10 mL chloroform were added and the solution heated to reflux. 231 mg of 4-hydroxybutyric acid, sodium salt and 20 mL chloroform were mixed in a 100 mL ehrlenmeyer flask. Two mL of sulfuric acid were added to this suspension and after 15 minutes, the butyric acid had dissolved. This solution was added in four aliquots to the refluxing oxo dye solution over two hours. Thin-layer chromatography revealed the dissappearance of blue-fluorescing starting material and the appearance of a new, blue-fluorescing spot. The reaction mixture was allowed to cool, and then neutralized with 1 M sodium hydroxide solution. The neutralized reaction mixture was then extracted via separatory funnel four times with 50 mL chloroform. The chloroform extracts were combined, dried over anhydrous sodium sulfate, filtered and the solvent removed via rotary evaporation. The remaining solid was purified via silica column chromatography using an eluent of 98% chloroform, 2% methanol.

EXAMPLE 15

Synthesis of an Amino-Functionalized Oligonucleotide

A modified thymine phosphoramidite (amino modifier dT) and a 5' amino linker (5' amino modifier C6) is obtained from Glen Research, Inc. (Sterling, VA). DNA oligomer is made on a PE Biosystems Nucleic Acid, Synthesizer Model No. ABI 3948 synthesizer using standard B-cyanoethyl phosphoramidite chemistry and incorporating either a modified thymine base with a terminal amino linker arm at a position within the sequence or an amino modifier at the 5 terminus of the oligomer.

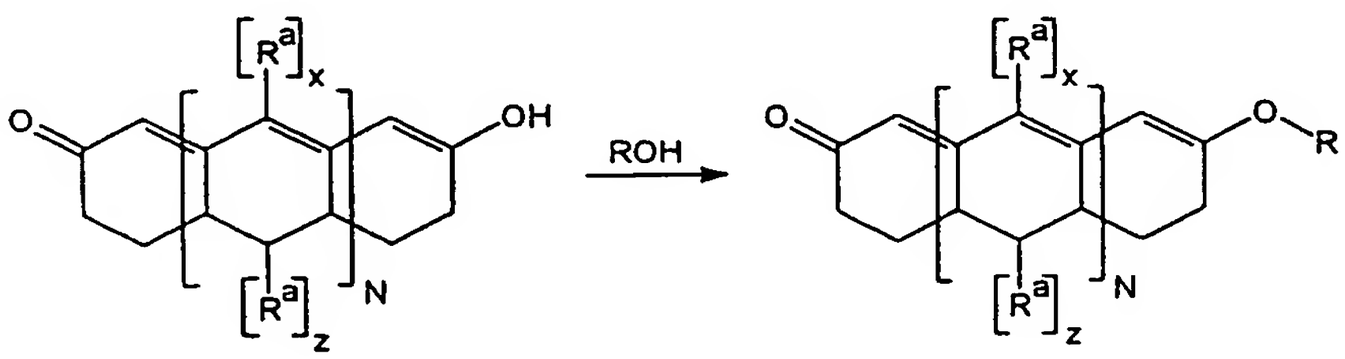
EXAMPLE 16

General protocol for synthesis of NHS-ester functionalized dye

In a 250 ml flask are placed 150 ml dry acetone or dioxane, 2.7 mmol carboxy-functionalized dye, 3.0 mmol N,N'-dicyclohexylcarbodiimide and 3.0 mmol N-hydroxysuccinimide. The flask is stirred overnight at room temperature in the dark. The precipitate is filtered off and discarded. The remaining solution is concentrated, 200 ml diethylether added, and then extracted twice with 150 ml 0.1 M sodium bicarbonate solution and twice with a saturated sodium chloride solution. The ether layer is dried with sodium sulfate, filtered and the solvent removed. The solid is washed with hexane and vacuum dried.

EXAMPLE 17

General procedure for the covalent attachment of a biological molecule containing a hydroxyl group to an oxo-dye



1mmol of the oxo dye in 5 mL chloroform are placed in a 50 mL round bottomed flask and mixed. To the heterogeneous mixture, 0.5 mL concentrated sulfuric acid are carefully added. An additional 10 mL chloroform are added and the solution is heated to reflux. 100 mmol of ROH and 20 mL chloroform are mixed in a separate 100 mL ehrlenmeyer flask where ROH is a linker, mono- or polysaccharide, amino acid, oligonucleotide, protein, glycoside or lipid, for example, which contains a reactive hydroxyl group; and R = alkyl, alkenyl, alkynyl, aryl and substituted versions thereof. Two mL of sulfuric acid are added to this suspension. This resulting solution is added in four aliquots to the refluxing oxo dye solution over two hours. The reaction mixture is allowed to cool, and then neutralized with 1 M sodium hydroxide solution. The neutralized reaction mixture is extracted via separatory funnel four times with 50 mL chloroform. The chloroform extracts are combined, dried over anhydrous sodium sulfate, filtered and the solvent removed via rotary evaporation. The remaining solid is purified via silica column chromatography.

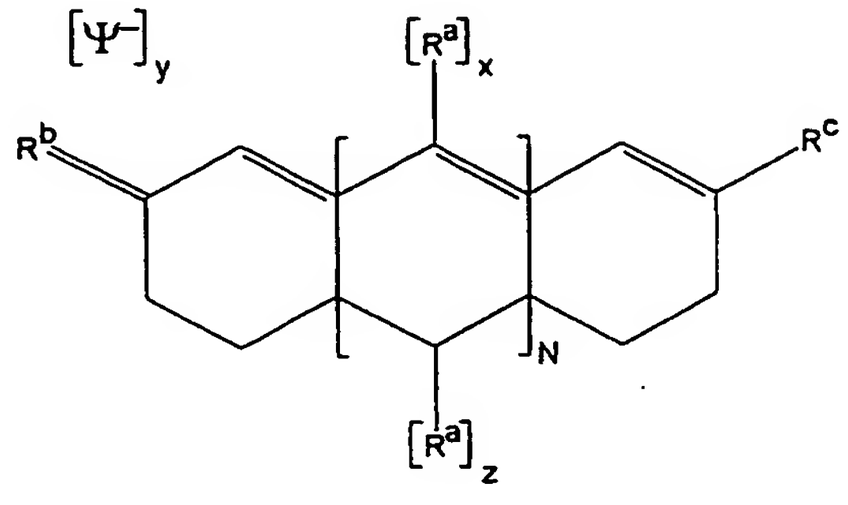
EXAMPLE 18

General protocol for labeling of 5′-amino-functionalized oligonucleotides, proteins, mono- or polysaccharides

An oligonucleotide, protein or aminosaccharide is dissolved in PBS buffer, pH 8, to a concentration of 10 nmol in 150 µl and the solution is vortexed. The Dye-Ester is dissolved in 50 µl *amine-free* DMF, using a five to 10 molar equivalent excess of dye to oligonucleotide (50 – 100 nmol ester). The DMF/ester solution is added to the oligonucleotide solution and vortexed. The reaction is allowed to occur for 12 hours at room temperature. Purification of the labeled biomolecule is done with an *Amersham Pharmacia Biotech* NAP-10 column.

WHAT IS CLAIMED IS:

1. A central plane-rigidized cyanine dye for labeling of biomolecules, said dye corresponding to the formula:



wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

2. The dye of claim 1 wherein R^b and R^c may be the same or different and are conjugated to said central plane-rigidized dye for altering wavelength emission of said dye.

3. The dye of claim 1 wherein one or more of R^a, R^b and R^c contain a polar moiety which increases the solubility of said dye.

4. The dye of claim 1 wherein one or more of groups R^a, R^b and R^c contain a linker portion between their attachment to the dye and said reactive substituent.

5. The dye of claim 1 wherein R^a is H or a substituted or unsubstituted aliphatic or aromatic group.

6. The dye of claim1 wherein R^a is H, a substituted or unsubstituted group selected from the classes consisting of alkyl, alkenyl, alkynyl, aryl and combinations thereof.

7. The dye of claim 1 wherein said reactive substituent forms a bond with at least one moiety selected from the group consisting of amino, hydroxy, thiol, carboxyl, aldehyde and ketone.

8. The dye of claim 1 wherein said reactive substituent is selected from the group consisting of alcohols, aldehydes, thiols, reactive esters, acids, acid halides, sulfonyl halides, hydrazines, ketones, haloacetamides, amides, and azides.

9. The dye of claim 1 wherein said substituent is selected from the group consisting of hydroxyl group, amido group, carboxylic group and amino group.

10. The dye of claim 1 wherein said substituent is selected from the group consisting of phosphoramidite, isothiocyanate, isocyanate, monochlorotriazine, dichlorotriazine, mono-or di-halogen substituted pyridine, mono-or di-halogen substituted diazine, aziridine, hydroxysuccinimide ester, hydroxysulfosuccinimide ester and imido ester.

11. The dye of claim 4 wherein said linker is a linear or branched, cyclic or heterocyclic, saturated or unsaturated group, each having C_{1-20}.

12. The dye of claim 11 wherein said linker includes one or more atoms selected from the group consisting of N, P, O and S.

13. The dye of claim 11 wherein said linker contains any combination of ether, thioether, amine, ester or amide bonds.

14. The dye of claim 11 wherein said linker contains one or more double or triple bonds.

15. The dye of claim 14 wherein said bonds include carbon-carbon, phosphorous – oxygen, phosphorous-sulfur, nitrogen-nitrogen, nitrogen-oxygen, aromatic or heteroaromatic bonds.

16. The dye of claim 3 wherein said polar moiety is selected from the group consisting of hydroxy, nitro, sulfonate, sulfate, carboxylate, substituted amine, quaternary amine and nitrile.

17. The dye of claim 1 wherein said R^b and R^c are selected from the group consisting of O, OR, $NR^1 R^2$ and $CR^3 R^4$, wherein R is H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, or mono- or polysaccharide; R^1, R^2 and R^4 are independently H,

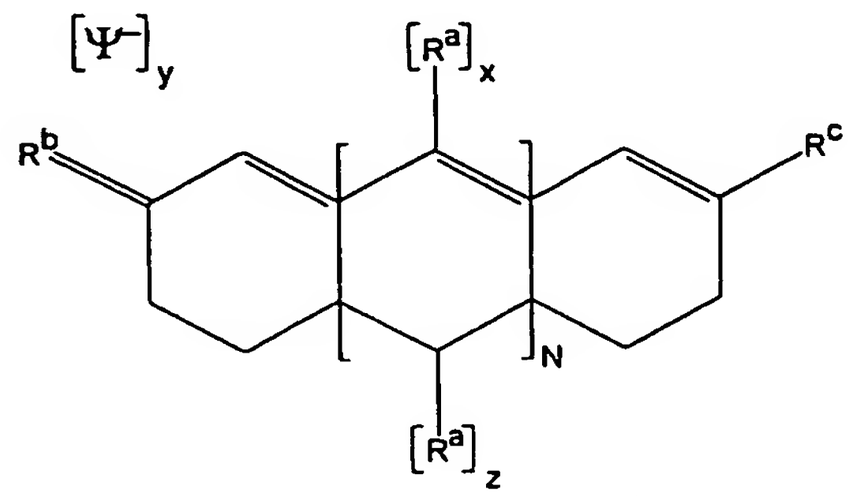
substituted or unsubstituted alkyl, alkenyl, alkynyl, aryl group, or contain a reactive substituent selected from the group consisting of phosphoramidite, isothiocyanate, isocyanate, monochlorotriazine, dichlorotriazine, mono-or di-halogen substituted pyridine, mono-or di-halogen substituted diazine, aziridine, sulfonyl halide, acid halide, glyoxal, reactive ester, aldehyde, alcohol, hydroxyl group, carboxylic group, amine, amide, thiol, amino group, sulfonyl halide, azides, acids, hydrazines, ketones, haloacetamides, hydroxysuccinimide ester, hydroxysulfosuccinimide ester and imido ester; R^3 is a ringed structure selected from the group consisting of indole, oxazole, thiazole, imidazole, quinoline, isoquinoline, aniline, carbazole, benzindole, benzoxazole, benzothiazole, benzimidazole, benzotriazole or substituted versions thereof; and R^1 and R^2 may join to form a ringed structure selected from the group consisting of indole, oxazole, thiazole, imidazole, quinoline, isoquinoline, aniline, carbazole, benzindole, benzoxazole, benzothiazole, benzimidazole, benzotriazole or substituted versions thereof.

18. The dye of claim 17 wherein said R^1, R^2, and R^4 may independently also contain a polar moiety selected from the group consisting of hydroxy, nitro, sulfonate, sulfate, carboxylate, substituted amine, quaternary amine and nitrile groups and alcohols.

19. The dye of claim 17 wherein said ringed structure contains a reactive substituent selected from the group consisting of phosphoramidite, isothiocyanate, isocyanate, monochlorotriazine, dichlorotriazine, mono-or di-halogen substituted pyridine, mono-or di-halogen substituted diazine, aziridine, sulfonyl halide, acid halide, glyoxal, reactive ester, aldehyde, alcohol, hydroxyl group, carboxylic group, amine, amide, thiol, amino group, sulfonyl halide, azides, acids, hydrazines, ketones, haloacetamides, and esters, hydroxysuccinimide ester, hydroxysulfosuccinimide ester and imido ester.

20. The dye of claim 17 wherein said ringed structure may also contain a polar moiety to increase solubility of said dye in aqueous solutions selected from the group consisting of hydroxy, nitro, sulfonate, sulfate, carboxylate, substituted amine, quaternary amine, nitrile groups and alcohols.

21. A fluorescent complex comprising a biological molecule which is bound either directly or indirectly to one or more dye molecules of the formula:



wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

22. A fluorescent complex according to claim 21 wherein functional groups are present either on the dye or the biological molecule for formation of said complex, said functional groups being selected from the group consisting of amines, amino group, carboxy group, hydroxy group, alcohols, thiols, acids, aldehydes and ketones.

23. A fluorescent complex according to claim 21 wherein said biological molecule is bound directly to said dyes via any combination of ether, thioether, amine, ester, amide or thiourea bonds.

24. A fluorescent complex according to claim 21 wherein said biological molecule is bound indirectly to said dye via a linking group.

25. The complex of claim 21 wherein the attachment of the dye to a biological molecule includes a nitrogen-containing group, a sulfur-containing group or oxygen-containing group.

26. A fluorescent complex according to claim 21 wherein the bond between said dye and said biological molecule is formed from the reaction of an activated ester of N-hydroxysuccinamide on the dye and a carboxyl group on the biological molecule.

27. A fluorescent complex according to claim 21 wherein the bond between said dye and said biological molecule is formed from the reaction of an activated phosphate ester of a phoshoramidite on the dye and a hydroxyl group on the biological molecule

28. A fluorescent complex according to claim 21 wherein the biological molecule is a protein.

29. A fluorescent complex according to claim 21 wherein the biological molecule is a nucleic acid polymer or nucleic acid base.

30. A fluorescent complex according to claim 21 wherein the biological molecule is a mono- or polysaccharide.

31. A fluorescent complex according to claim 29 wherein said nucleic acid polymer is a natural or synthetic oligonucleotide.

32. A fluorescent complex according to claim 29 wherein said nucleic acid polymer is a chromosome.

33. A fluorescent complex according to claim 29 wherein said nucleic acid polymer is DNA.

34. A fluorescent complex according to claim 29 wherein said nucleic acid polymer is RNA.

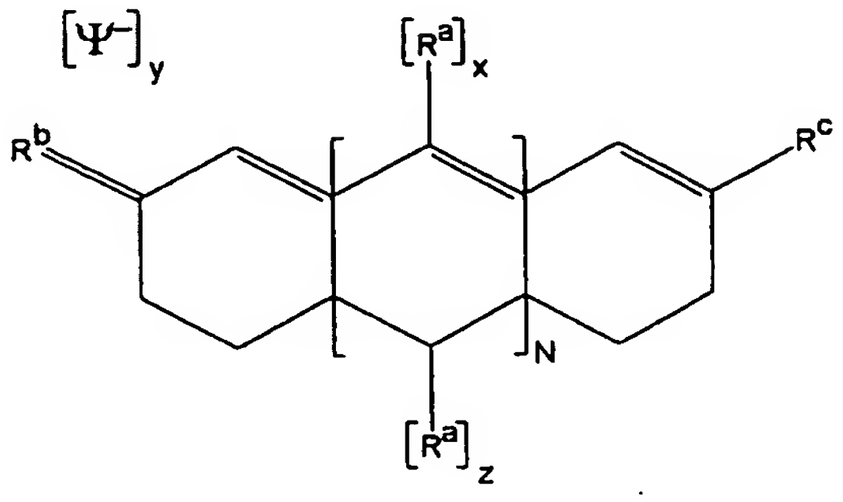
35. A fluorescent complex according to claim 28 wherein said protein is an antibody.

36. A fluorescent complex according to claim 21 wherein the biological molecule is a cell.

37. A fluorescent complex according to claim 21 wherein the biological molecule is a lipid.

38. A method of detecting a target biological molecule comprising:

(a) combining a sample that contains or is thought to contain a target molecule with a dye of the formula:



wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

(b) incubating the combined sample and dye compound for a time sufficient for the dye compound to combine with said target molecule in the sample to form one or more dye-target molecule complexes that give a detectable fluorescent signal; and

(c) detecting said signal.

39. The method of claim 38 wherein said target molecule is selected from the group consisting of nucleic acid polymers, nucleic acid bases, proteins, lipids, cells, tissue specimens, and polysaccharides.

40. The method of claim 38 wherein said target molecule is DNA or RNA.

41. The method of claim 38 wherein said target molecule is a natural or synthetic oligonucleotide or oligonucleoside.

42. The method of claim 38 wherein said target molecule is a chromosome.

43. The method of claim 38 wherein said target molecule is adenine, thymine, cytosine, guanine, nitrogen heterocyclic bases and derivatives thereof.

44. The method of claim 38 further comprising the step of adding one or more reagents to the sample prior to or during said step of combining, where each additional reagent is capable of a response that is detectably different from said dye-target molecule complex.

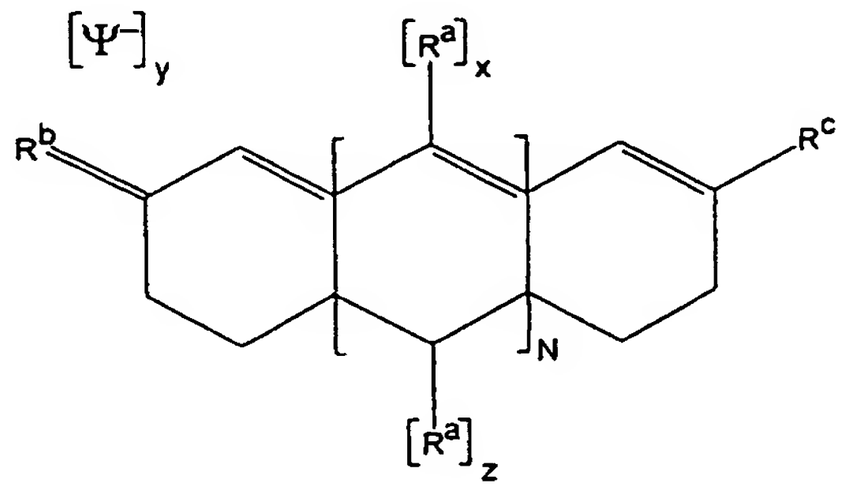
45. The method of claim 38 further comprising the step of adding one or more reagents to the sample during said step of incubating where each additional reagent is capable of a response that is detectably different from said dye-target molecule complex.

46. The method of claim 44 wherein said further detection reagent is selected from the group consisting of antibodies, oligonucleosides and oligonucleotides

47. The method of claim 45 wherein said further detection reagent is selected from the group consisting of antibodies, oligonucleosides and oligonucleotides.

48. A method of detecting a target molecule comprising:

(a) providing a probe wherein said probe is a fluorescent complex formed from a reaction of said probe with a dye molecule of the formula:



wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

(b) combining a sample that contains or is thought to contain a target molecule with said probe;

(c) incubating the combined sample for a time sufficient to allow said target molecule

to bind to said probe to form one or more dye-probe-target molecule complexes that give a detectable signal;

(d) detecting said signal.

49. The method of claim 48 wherein said target molecule is selected from the group consisting of nucleic acid polymers, nucleic acid bases, proteins, lipids, cells, tissue specimens, and mono- or polysaccharides.

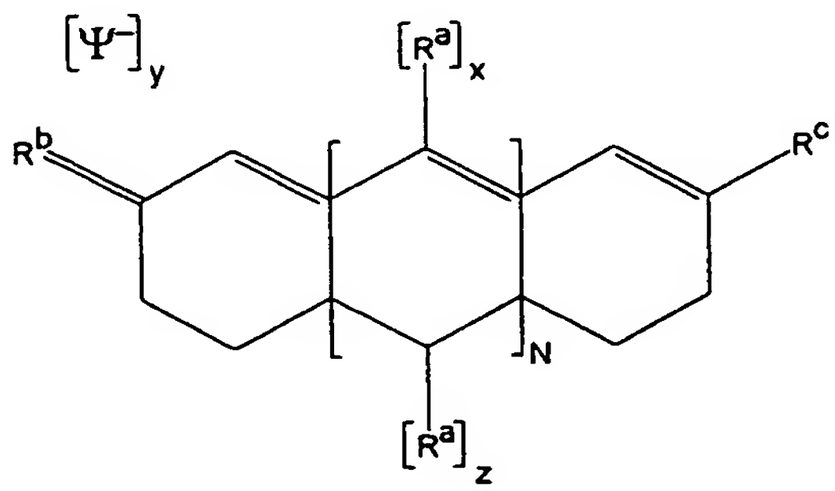
50. The method of claim 48 wherein said target molecule is DNA or RNA.

51. The method of claim 48 wherein said probe is an antibody.

52. The method of claim 48 wherein said probe is an oligonucleotide.

53. A method of characterizing a nucleic acid sample comprising:

(a) providing one or more fluorescent complexes comprising one or more biological molecules bound to at least one dye compound of the formula :



wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

(b) combining said nucleic acid sample with said fluorescent complexes;

(c) incubating said nucleic acid sample with said fluorescent complexes under

reaction conditions which allow said fluorescent complexes to combine with or incorporate within said nucleic acid to form a staining profile having detectable fluorescent signals, said staining profile being characteristic of the sample; and

(d) characterizing said nucleic acid based on said staining profile.

54. The method according to claim 53 wherein said biological molecules are natural, synthetic or modified nucleotides, nucleosides, oligonucleotides, oligonucleosides, DNA, RNA or proteins.

55. The method according to claim 53 wherein the sample is a solution comprising nucleic acid polymers separatable by means of relative mobility.

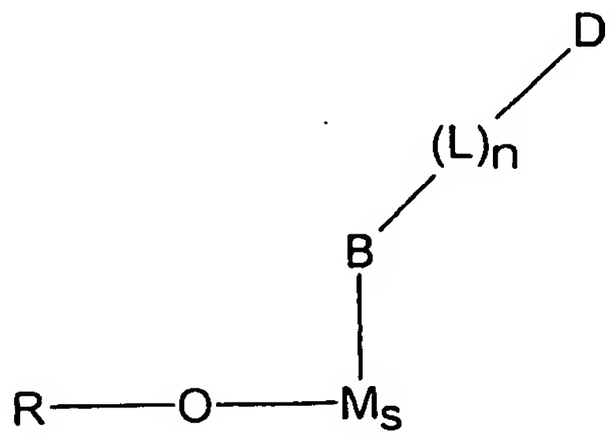
56. The method of characterizing a sample according to claim 53, further comprising adding one or more additional reagents to the sample, where each additional reagent is capable of a response that is detectably different from the fluorescent signal of said dye-nucleic acid complex.

57. The method of claim 53 wherein said characterization is with respect to purity of the solution, composition of polymers in solution and composition of components present in solution.

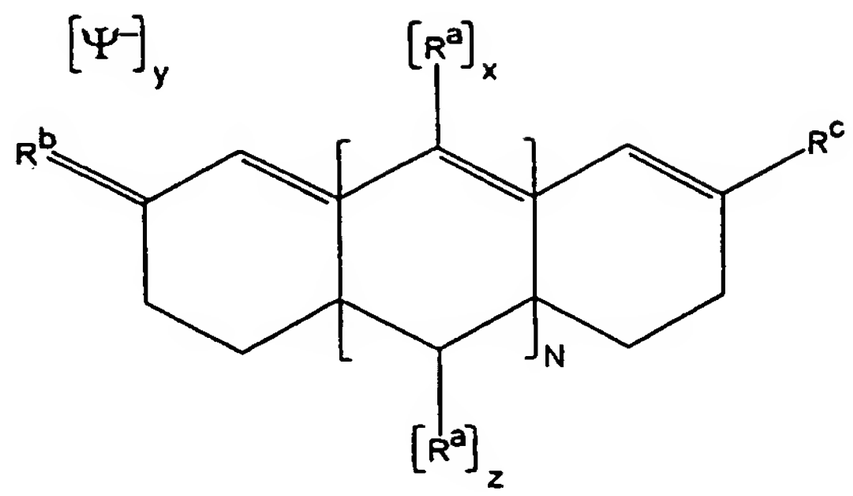
58. The method of characterizing a sample according to claim 56, wherein the additional detection reagent is an antibody.

59. The method of characterizing a sample according to claim 53 wherein said nucleic acid sample is DNA or RNA.

60. A chemical complex of the formula:



wherein D is of the formula:



wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

L is a linear, branched, cyclic or heterocyclic, saturated or unsaturated group, each having $C_{1\text{-}20}$; n is 0 or 1; R-O-M_s-B is a natural or modified nucleotide or nucleoside inclusive of DNA chain terminators wherein R is selected from the group consisting of H, PO_3^{2-}, $P_2O_6^{3-}$, $P_3O_9^{4-}$, alpha –thiophosphates, alpha BH_3-phosphates and derivatives thereof; O is an oxygen molecule; M_s is a sugar residue; and B is a nitrogen-containing heterocyclic base;

61. The complex of claim 60 wherein M_s is selected from the group consisting of ribosyl, 2'-deoxyribosyl, 3'-deoxyribosyl, 2', 3'-dideoxyribosyl, 2'-oxabutyl ribosyl, 2'-oxamethyl ribosyl and other modified ribose sugars.

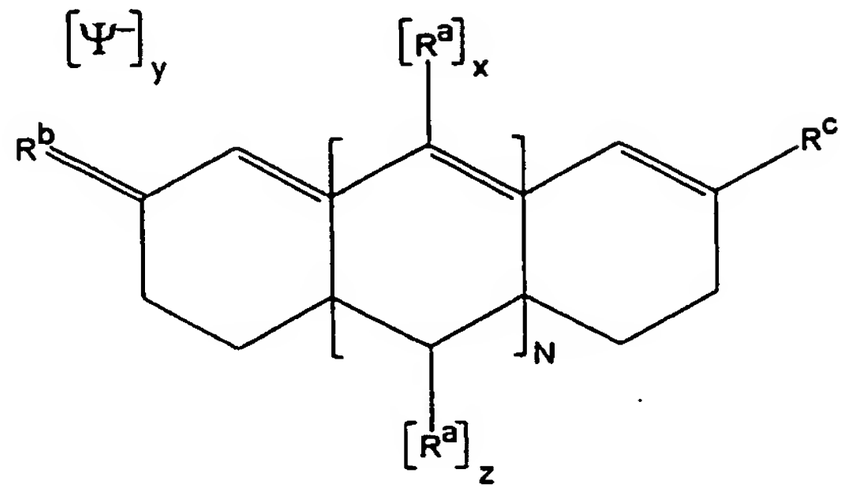
62. The complex of claim 60 wherein B is selected from the group consisting of uracil, thymine, cytosine, guanine, 7-deazaguanine, hypoxanthine, 7-deazahypoxanthine, adenine, or 7-diazaadenine, 2,6-diaminopurine and other nitrogen-heterocycle bases.

63. The method of labeling a nucleic acid molecule comprising the step of incorporating the complex of claim 60 into a nucleic acid chain.

64. The method of claim 63 further comprising the step of determining the nucleic acid sequence of the molecule.

65. A labeling kit comprising:

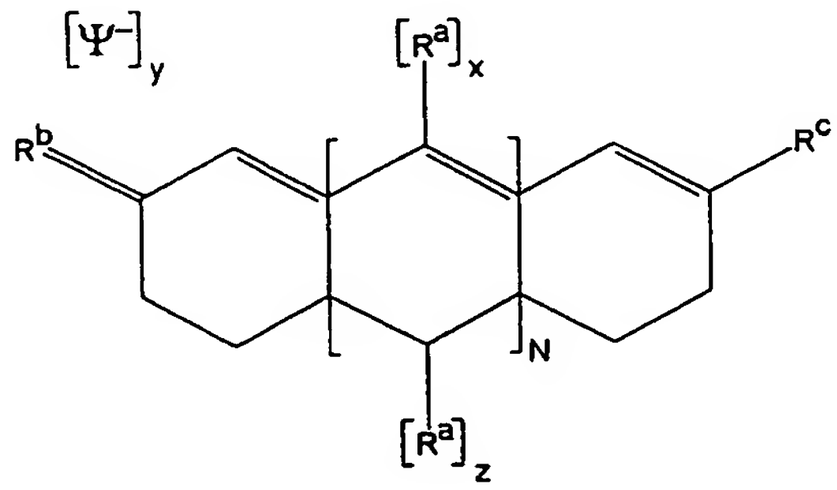
(a) at least one dye compound of the formula:



wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

(b) solvent or buffer suitable for chemical labeling of a biological molecule with said dye.

66. A DNA sequencing kit comprising a mixture containing dideoxynucleotide chain terminators wherein said terminators are each independently bound to a dye of the formula:

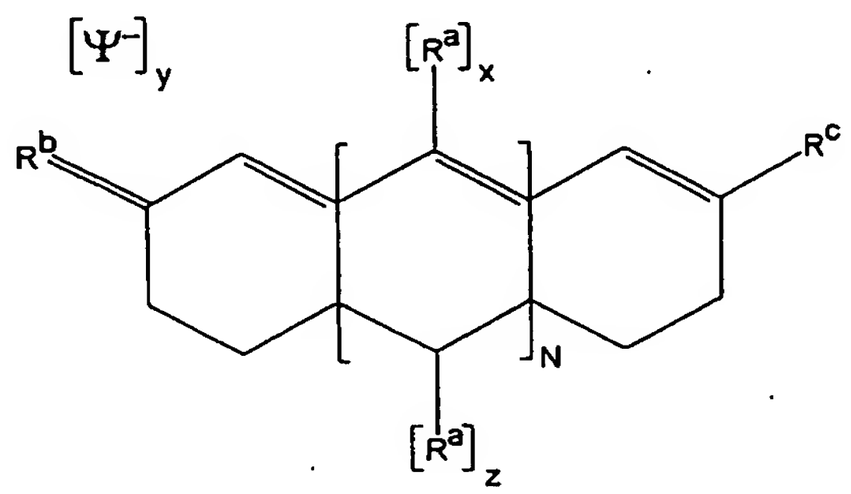


wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

67. The kit of claim 66 wherein each terminator provided in said mixture has bound to it a dye with an emission wavelength which is different from that of the other terminators in the mixture.

68. A DNA sequencing kit comprising:

(a) a mixture containing dideoxynucleotide triphosphate chain terminators wherein said terminators are each independently bound to a dye of the formula:



wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

(b) an oligonucleotide primer complimentary in sequence to a region of a vector template suitable for DNA sequencing or to a region of a DNA to be copied;

(c) DNA polymerase;

(d) a PCR buffer;

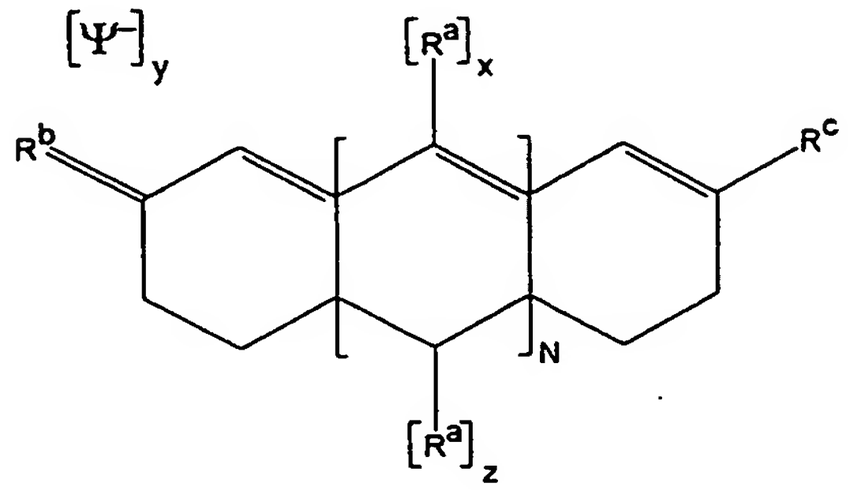
(e) deoxynucleotide triphosphates; and

(f) a vector suitable for DNA sequencing.

69. The kit of claim 68 wherein each terminator provided in said mixture has bound to it a dye with an emission wavelength which is different from that of other terminators in the mixture.

70. A DNA sequencing kit comprising:

(a) an oligonucleotide primer bound to a dye of the formula:



wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

(b) deoxynucleotide triphosphates;

(c) dideoxynucleotide triphosphate chain terminators;

(d) a PCR buffer;

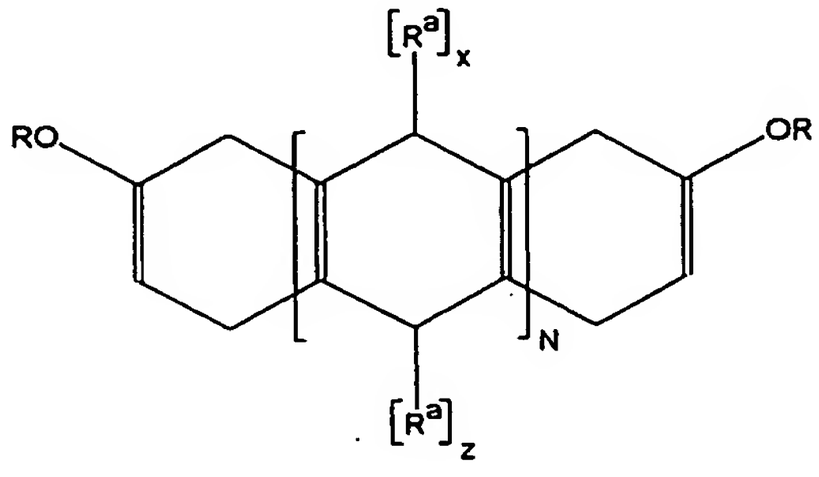
(e) DNA polymerase; and

(f) a vector suitable for DNA sequencing.

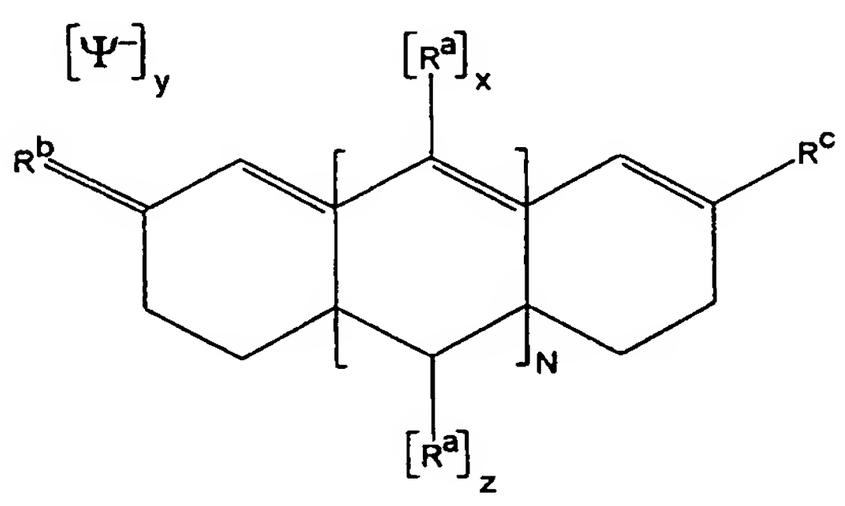
71. The kit of claim 70 wherein the dye-bound oligonucleotide primer is complementary in sequence to a region of a vector suitable for DNA sequencing or to a region of a DNA to be copied.

72. A method of synthesizing a plane-rigidized dye comprising:

reacting a compound corresponding to the structure:



with a suitable reactant or reagent to form the compound:



wherein N=1-4; Ψ^- is a compatible counterion; y = from 0 to 1; x = 0 or 1; z = 0 or 1; R is methyl or akyl C_{1-6}, and wherein at least one of R^a, R^b or R^c is a group containing a reactive substituent for bonding of said dye to said biological molecule.

73. A method of claim 72 wherein said suitable reactant or reagent alters the wavelength emission of said dye through the addition of at least one end group.

74. A method of claim 72 wherein said suitable reactant or reagent comprises aliphatic or aromatic hydrocarbons or heterohydrocarbons having C_{1-40} containing a methyl group having acidic hydrogens.

75. A method of claim 74 wherein said methyl group is within 2, 4 or 6 carbons of a nitrogen atom.

76. A method of claim 72 further including the step of reacting the resultant dye with a protein, nucleic acid polymer, nucleic acid base, mono- or polysaccharide, or cell.

77. A method of claim 72 wherein said suitable reactant or reagent is an acid.

78. A method of claim 72 wherein said dye is formed from an acid-catalyzed addition of two end groups followed by a rearrangement.

79. A method of making a partially reduced linear polyarene compound useful as a dye precursor comprising the steps of:

(i) providing an aryl alkali metal compound having alkoxy and carboxylate moieties;

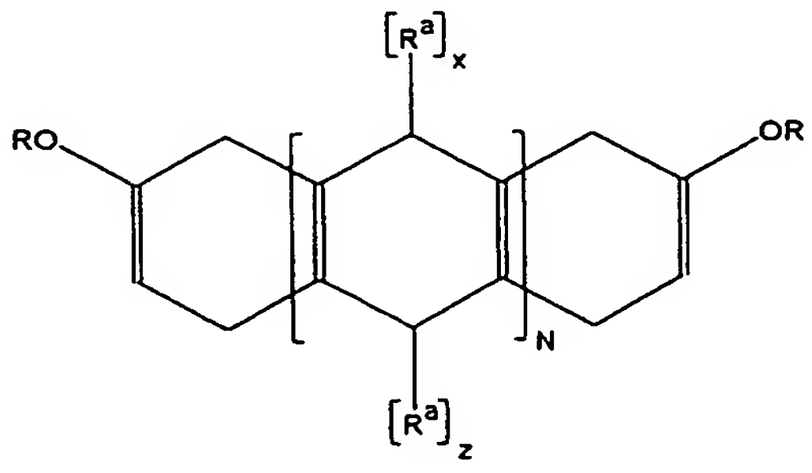
(ii) reacting said compound with an alkoxy aryl acid halide to form a keto-acid;

(iii) reacting said keto-acid under conditions sufficient to produce a closed-ring quinone structure;

(iv) reducing said resultant quinone structure to form a linear polyarene;

(v) optionally functionalizing said linear polyarene at position R^a; and

(vi) performing a dissolving metal reduction step to produce a compound corresponding to the structure:



wherein R is methyl or alkyl C_{1-6} and R^a optionally contains a reactive functional group for bonding to a biological molecule, x is 0 or 1 and z is 0 or 1; and N is 1-4.

80. The method of claim 79, wherein said aryl alkali metal compound is lithium-2-lithio-5-methoxybenzoate or lithium-3-lithio-7-methoxy-2-naphthoate.

81. The method of claim 79, wherein said alkoxy aryl acid halide is *p*-methoxybenzoylchloride, 6-methoxy-2-naphthoylchloride, isophthaloyldichloride or naphthalene-2,7-dicarbonyl chloride.

82. The method of claim 79, wherein said keto-acid is 2-(-4-methoxybenzoyl)-3-methoxybenzoic acid, 2-(6-methoxy-2-naphthoyl)-3-methoxybenzoic acid, 1,3-*bis*-(2-carboxy-4-methoxybenzoyl)-benzene or 2,7-*bis*-(2-carboxy-4-methoxybenzoyl)-naphthalene.

83. The method of claim 79, wherein said quinone is 2,7-dimethoxyanthraquinone, 2,9-dimethoxy-5,12-naphthacenedione, 2,10-dimethoxy-6,13-pentacenedione, 2,10-dimethoxy-5,7,12,14-pentacenetetraone or 2,11-dimethoxy-5,8,13,16-hexacenetetraone.

84. The method of claim 79, wherein said linear polyarene of step (iv) is 2,7-dimethoxyanthracene, 2,9-dimethoxynaphthacene, 2,10-dimethoxypentacene and 2,11-dimethoxyhexacene.

85. The method of claim 79, wherein said partially reduced linear polyarene compound is 2,7-dimethoxy-1,4,5,8,9,10-hexahydroanthracene, 2,9-dimethoxy-1,4,5,6,7,10,11,12-octahydronaphthacene, 2,10-dimethoxy-1,4,5,6,7,8,11,12,13,14-decahydropentacene, or 2,11-dimethoxy-1,4,5,6,7,8,9,12,13,14,15,16-dodecahydrohexacene.

86. The method of claim 79, further comprising the step of attaching said precursor dye to a biomolecule.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/07783

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/68; C12P 19/34
US CL :435/6, 91.1, 91.2; 536/22.1, 25.32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.2; 536/22.1, 25.32

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN
search terms: cyanine dyes, oligonucleotide, DNA sequencing

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,332,666 A (PROBER et al.) 26 July 1994, see entire document.	1-86
Y	US 4,600,775 A (THEODOROPULOS) 15 July 1986, see entire document.	1-86
Y	US 5,187,085 A (LEE) 16 February 1993, see entire document.	1-86

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
26 JUNE 2000	31 JUL 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEZIA RILEY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/07783

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/07783

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-20, 38-47, AND 72-78, drawn to a cyanine dye.
Group II, claim(s) 21-37 AND 48-59, drawn to fluorescent complex.
Group III, claim(s) 60-65, drawn to chemical complex.
Group IV, claim(s)66-71, drawn to a sequencing kit.
Group V, claim(s) 79-86, drawn to a method of making polyarene.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: Group I-V correspond to different special technical features.